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Karolinska Institutet, Stockholm, Sweden

# **INTRATUMORAL HETEROGENEITY IN GLIOBLASTOMA: SUBTYPE TRANSITION AND CELL-TO-CELL COMMUNICATION**

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# **INTRATUMORAL HETEROGENEITY IN GLIOBLASTOMA: SUBTYPE TRANSITION AND CELL-TO-CELL COMMUNICATION**

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*Dedicated to my beloved family*



## ABSTRACT

Glioblastoma is a most aggressive brain tumor with a median overall survival of less than two years. The current treatment is surgery, chemotherapy, and radiotherapy. However, glioblastoma is difficult to treat and recurs some time after treatment. Several reports have shown that intratumoral heterogeneity is a hallmark of glioblastoma, and it is often said to be a factor that contributes to tumor progression and tumor relapse.

Based on the single-cell gene expression profiling, glioblastoma is found to be composed of tumor cells with three different subtype signatures namely proneural, classical, and mesenchymal. These subtypes may transition between each other, which may be affected, and/or potentially controlled, by genetic and epigenetic modifications. Even microenvironmental events and communication between different subtype cells within the same tumor may play a role. Studies in this thesis focused on identifying regulators of subtype transitions and their involvement in cell-to-cell communication in individual glioblastomas.

Study I used several analytical approaches including connectivity map analysis, overexpression screen, RNA-sequencing, and gene expression profiling. SOX2 was identified to have the capacity to transition mesenchymal gene expression subtype glioblastoma cells back to a non-mesenchymal signature. Subsequently, *SFRP2* was identified as a SOX2 antagonist able to induce a mesenchymal transition. In line with this, *SFRP2* was found expressed at a higher level in mesenchymal as compared to proneural and classical TCGA glioblastoma samples. *In vitro*, *SFRP2* decreased tumor sphere formation and cell proliferation, and increased cell invasion capacity and sensitivity towards apoptotic stimuli. Spatial gene expression analysis found *SFRP2*-correlated genes to be expressed in a higher level in vascularized areas in glioblastoma, as opposed to *SOX2*-correlated genes that were highly expressed in cellular tumor regions. Moreover, conditioned media from *SFRP2* transitioned cells induced more *MRC1* (encodes CD206) expression in human monocytes *in vitro*. Collectively, these experiments identified *SFRP2* as a SOX2 antagonist and inducer of mesenchymal transition in glioblastoma.

In Study II *PROX1* was found expressed at a lower level in glioblastoma as compared to low-grade gliomas. Tumors with lower *PROX1* correlated with a mesenchymal subtype and patients exhibited shorter survival. Suppression of *PROX1* in glioblastoma cell lines with high *PROX1* levels induced transition to a mesenchymal glioblastoma subtype signature. Conversely, overexpression of *PROX1* transitioned cells to a non-mesenchymal subtype signature. In co-occurrence with these transitions, *PROX1* functionally impacted on cell proliferation and levels of several cell cycle proteins. *SOX2* was identified as an upstream signaling component of *PROX1*, and thus *SOX2* and *PROX1* levels decreased upon treatment with a CDK inhibitor. By co-immunoprecipitation experiments *PROX1* was found

to interact with THRAP3 in the nucleus. Depletion of THRAP3 increased PROX1 expression and protein stability. Based on previous reports on THRAP3, these findings suggest THRAP3 to be involved in the transcriptional regulation of PROX1. These results underscore a functional role of PROX1 in glioblastoma development and with implications for survival outcome.

In study III the well-known U-343 cell line system was used as a model system to study intratumoral heterogeneity and cell-to-cell communication. The four cell lines U-343 MG, U-343 MGa, U-343 MGa 31L and U-343 MGa C12:6 are derived from the same glioblastoma, and display different phenotypes including marker expression, cells shape, and proliferation. Gene expression and DNA copy number analyses suggested their shared derivations from a common ancestor in a tumor evolutionary relationship. Temozolomide (TMZ) sensitivity of the individual cell lines was determined. In a cell culture composed of a mixture of all four cell lines, the one with the lowest sensitivity outlived the others during TMZ treatment, which modeled the appearance of drug resistance. Finally, co-culture and conditioned media experiments revealed complex interactions between the U-343 cell lines through signaling by cell-to-cell contact or secreted proteins. These findings provide a model for research on intratumoral heterogeneity, both with regard to appearance of drug resistance and to interclonal interactions.

Collectively, these studies provide insights into the complexity of intratumoral heterogeneity, including subtype transitions during tumor progression and interclonal communication. Intratumoral heterogeneity is thus important to consider when designing new therapeutic strategies and may also in itself pose novel therapeutic targets.

**Keywords:** glioblastoma, intratumoral heterogeneity, subtype transition, intercellular communication, U-343, SOX2, SFRP2, PROX1, THRAP3, TGFBI, NOTCH1, and ADAMTS1



## LIST OF SCIENTIFIC PAPERS

**I. Min Guo**, Kaveh M. Goudarzi, Shiva Abedi, Jinan Behnan, Melanie Pieber, Elin Sjöberg, Xing-Mei Zhang, Patrik Ernfors, Robert A. Harris, Jiri Bartek, Mikael S. Lindström, Monica Nistér, Daniel Hägerstrand

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**II.** Kaveh M. Goudarzi, Jaime A. Espinoza, **Min Guo**, Jiri Bartek, Monica Nistér, Mikael S. Lindström, Daniel Hägerstrand

**Reduced expression of PROX1 transitions glioblastoma cells into a mesenchymal gene expression subtype** *Cancer Research. 2018 Oct 15;78(20):5901-5916*

**III. Min Guo**, Marjolein Van Vliet, Jian Zhao, Teresita Díaz de Ståhl, Mikael S. Lindström, Huaitao Cheng, Susanne Heller, Monica Nistér<sup>†</sup>, Daniel Hägerstrand<sup>†</sup>

**Identification of functionally distinct and interacting cancer cell subpopulations from a glioblastoma with intratumoral genetic heterogeneity** *Neuro-Oncology Advances. 2020 May 27; 2(1), vdaa 061*

<sup>†</sup> These authors share senior responsibility.

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**V.** Hao Ni, **Min Guo**, Xuepei Zhang, Lei Jiang, Shuai Tan, Juan Yuan, Huanhuan L Cui, Yanan Min, Junhao Zhang, Susanne Schlisio, Chunhong Ma, Wangjun Liao, Monica Nistér, Chunlin Chen, Shuijie Li, Nailin Li

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## LIST OF ABBREVIATIONS

ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motif 1
ALDH3A1	Aldehyde dehydrogenase isoform 3A1
AP1	Activator protein 1
APC	Antigen-processing cell
ASCL1	Achaete-scute homolog 1
BBB	Blood-brain-barrier
BDNF	Brain derived neurotrophic factor
BMAL1	Brain and muscle Arnt-like protein 1
BMDM	Bone-marrow derived monocyte
BMP4	Bone morphogenetic protein 4
BTB	Blood-tumor-barrier
CCL	C-C motif chemokine ligand
CCLE	Cancer cell line encyclopedia
CDK	Cyclin-dependent kinase
CGH	Comparative genomic hybridization
ChIP	Chromatin immunoprecipitation
CK1	Casein kinase 1
CLOCK	Circadian locomotor output cycles kaput
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine
CRD	Cysteine-rich domain
CSC	Cancer stem cell
CSF	Colony stimulating factor
CT	Computed tomography
CTC	Circulating tumor cell
CTLA	Cytotoxic T lymphocytes associated molecule
DAAM	Disheveled-associated activator of morphogenesis
DCV	Dendritic cell vaccine
DKK	Dickkopf WNT signaling pathway inhibitor
DNMT	DNA methyltransferase
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
EV	Extracellular vesicle
EZH2	Enhancer of zeste homolog 2
FGF	Fibroblast growth factor
FOXM1	Forkhead box protein M1
FZD	Frizzled receptor
G-CIMP	Glioma CpG island methylator phenotype
GDNF	Glial cell-derived neurotropic factor
GFAP	Glial fibrillary acidic protein
GIC	Glioma initiation cell
GLUT1	Glucose transporter 1
GSC	Glioma stem cell
HB-EGF	Heparin binding epidermal growth factor

HDACi	Histone deacetylase inhibitor
HGCC	Human glioblastoma cell culture
HGF	Hepatocyte growth factor
HIF1 $\alpha$	Hypoxia-inducible factor 1 $\alpha$
IDH1/2	Isocitrate dehydrogenases 1/2
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iPSC	Induced pluripotent stem cell
LAG3	Lymphocyte activating 3
LIF	Leukemia Inhibitory Factor
MCP	Monocytes chemo-attractant protein
MRI	Magnetic Resonance Imaging
MSC	Mesenchymal stem cell
NADP+	Nicotinamide adenine dinucleotide phosphate
NDP	Norrin disease protein
NES	Nuclear export signal
NF	Neurofibromin
NF- $\kappa$ B	Nuclear factor kappa B
NFAT	Nuclear factor of activated T cell
NICD	Notch intracellular domain
NLD	Nuclear localization domain
NLS	Nuclear localization signal
NPC	Neural progenitor cell
NRF2	NF-E2-related factor 2
NSC	Neural stem cell
OPC	Oligodendrocytes precursor
OXPPOS	Oxidative phosphorylation
PcG	Polycomb group proteins
PCNA	Proliferating cell nuclear antigen
PD-1	Programmed Death 1
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol-3-kinase
PKG	Protein kinase G
PLC	Phospholipase C
PMT	Proneural to mesenchymal transition
PROX1	Prospero-related homeobox 1
PTEN	Phosphatase and tensin homologue
RB	Retinoblastoma
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
RPTP	Receptor-type protein tyrosine phosphatase
RTK	Receptor tyrosine kinase
SDF	Stromal-derived factor
SFRP	Secreted frizzled-related protein
SHH	Sonic hedgehog
SOX2	SRY (sex determining region Y)-box 2

SPECS	Secretome protein enrichment with click sugars analysis
STAT3	Signal transducer and activator of transcription 3
SVZ	Subventricular zone
TAA	Tumor-associated astrocyte
TAD	Transactivation domain
TAM	Tumor-associated macrophages/microglia
TAZ	Transcriptional activator with PDZ-binding motif
TBX6	T-box Protein 6
TCF/LEF	T-cell factor/lymphoid enhancer factor
TCGA	The Cancer Genome Atlas
TEAD	Transcriptional enhanced associated domain
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
TGFBI	Transforming growth factor beta induced
TIC	Tumor initiation cell
TIL	Tumor-infiltrating lymphocyte
TIM3	T cell immunoglobulin 3
TMZ	Temozolomide
TNF	Tumor necrosis factor
TPC	Tumor propagating cell
TTFields	Tumor Treating Fields
VEGF	Vascular endothelial growth factor
YAP	Yes-associated protein
$\alpha$ -KG	$\alpha$ -ketoglutarate
2-HG	2-hydroxyglutarate
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine

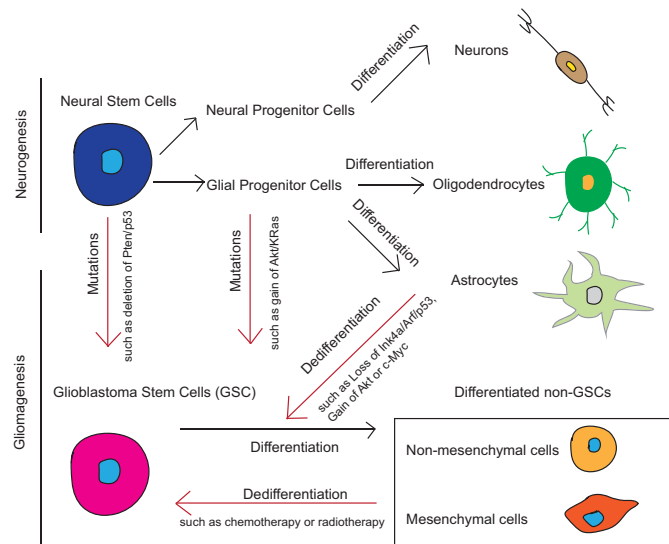
# 1. GLIOBLASTOMA INTRODUCTION

Glioblastoma, also known as glioblastoma multiforme, is the most aggressive glioma and accounts for 60% of all malignant primary brain tumors in adults. Glioblastoma has high morbidity and mortality, with a median overall survival of 12-18 months. It was reported that the incidence is higher in whites than Asians, and also more frequent in men than women by 1.58 times [1]. There are several risk factors for developing glioblastoma, including environmental and patients' intrinsic factors. Prior exposure to ionizing radiation is one established risk to develop glioblastoma [2].

Glioblastoma can be further classified as primary or secondary glioblastoma correlated with absence or presence of isocitrate dehydrogenases 1 and 2 (*IDH1/2*) mutations. Majority of glioblastomas are primary with wild-type *IDH1/2*, and arise from non-neoplastic cells, such as neural stem cells, glial progenitors or differentiated cells such as astrocytes [3]. Compared to secondary glioblastoma, primary glioblastoma has a worse prognosis even after intensive treatment. Patients with older age (>60 years) have an even shorter survival. Secondary glioblastoma often harbors *IDH1* or *IDH2* mutation, progresses from low-grade glioma and has a better prognosis [4, 5].

## 1.1 GLIOMAGENESIS

Three theories have been proposed about the origin of primary glioblastoma, namely from the glioblastoma stem cells (GSCs), or via dedifferentiation from progenitor cells, or from astrocytes (Figure 1).



**Figure 1. Relation between neurogenesis and gliomagenesis.** In the normal neurogenesis, neural stem cells (NSCs) give rise to neural- and glial-progenitors. Then neural progenitors differentiate into neurons, and glial progenitor cells differentiate into oligodendrocytes and astrocytes. However, during gliomagenesis, mutations that induce activation of oncogenic genes or loss of suppressive genes can happen in neural stem cells, progenitors or astrocytes, resulting in the generation of glioma stem cells (GSCs). GSCs can differentiate into non-mesenchymal and mesenchymal glioma cells and form tumor. On the other hand, some therapy stimuli can induce glioma cells dedifferentiate into GSCs.

Neural stem cells (NSCs) are reported as a source of glioblastoma initiation. NSCs are a small population of cells residing in neurogenic regions such as the subventricular zone (SVZ) and the hippocampus. NSCs give rise to neuroblasts and glial progenitor cells, which then develop to mature neurons, astrocytes or oligodendrocytes. GSCs share similar properties with NSCs, such as tumor sphere formation, and treatment resistance. In addition, glioblastomas are usually localized in the SVZ, making it reasonable that GSCs come from NSCs. Since many gene alterations exist in glioblastoma, it is hard to know if these alterations work singly or cooperatively during the transformation. Wang et al. have shown that *Tp53* in-frame deletion mutation in neural stem cells, by using hGFAP-Cre/*Tp53*<sup>flox/flox</sup> mouse model, induced accumulation of oncogenic alterations in retinoblastoma (RB) and receptor tyrosine kinase (RTK) pathways, and promoted gliomagenesis [6]. Moreover, different mutations in the adult stem cells led to different types of brain tumors. Deletion of *PTEN/Tp53* in adult sub-ventricular stem cells generated gliomas, although the deletion of *Rb/Tp53* and *Rb/Tp53/PTEN* induced primitive neuroectodermal tumors (PNET) [7].

Progenitor cells and astrocytes may also be considered as the origins of malignant glioma. It has been shown that the transfer of activated forms of *Akt* and *KRas* into Nestin-expressing neural progenitor cells led to tumor development, although this was ineffective in GFAP-expressing astrocytes. *Ink4a/Arf* are tumor suppressors that can activate *Rb* and *p53*. The loss of *Ink4a/Arf* was shown to enhance the capacity of *KRas* and *Akt* in the progenitor cells to form tumors. Moreover, the combination of *Ink4a-Arf* loss and *KRas/Akt* activation was effective in astrocytes and increased the incidence of tumor formation [8]. This indicates that oncogene activation and suppressor loss are important in the dedifferentiation of progenitors and astrocytes.

## 1.2 DIAGNOSIS

Glioblastoma occurs more frequently in the frontal lobe, compared to temporal and parietal lobes, mostly with headache and seizure symptoms. The current diagnosis, surveillance and therapeutic monitoring mainly depend on computed tomography (CT) and magnetic resonance imaging (MRI) scan, and histological examination.

MRI scan is a primary tool for glioblastoma diagnosis. MRI provides higher resolution multi-planar structural information. After MRI scanning, histological examination provides a definite diagnosis of glioblastoma. The tissue is collected after surgery, fixed, paraffin embedded, and stained with H&E as well as for some molecular biomarkers. As the name of multiforme suggests, glioblastoma is characterized by the presence of poorly differentiated pleomorphic astrocytic cells with frequent nuclear atypia and mitotic figures. There is often enhanced neo-angiogenesis, necrosis and pseudopalisading necrosis. Macroscopically, the central area is often yellowish necrosis from myelin breakdown, and is surrounded by peripheral grayish tumor cell areas, with multiple old and recent hemorrhages [9].



### 1.3 THERAPEUTIC APPROACHES

Current therapeutic approaches for glioblastoma are surgical resection, followed by adjuvant radiotherapy and chemotherapy, as well as treatment with alternating electrical fields [10].

*Surgical resection* plays an important role in both the diagnosis and treatment of the glioblastoma by reducing the tumor load and providing tissues for histological and molecular diagnosis. Nowadays, more advanced techniques have been developed to maximize extension of resection and optimize the safety during surgery, such as operation by using intraoperative MRI or ultrasound, electrophysiological monitoring by using electrodes, functional brain mapping by awake brain mapping techniques, and maximizing tumor visualization by using fluorescent markers such as 5-aminolevulinic acid under blue light, or fluorescein in white light.

*Radiation therapy* has improved the patients' survival in glioblastoma [11]. The treatment is usually initiated 3 to 4 weeks after surgery and lasts for 6 weeks, with a cumulative absorbed dose of 60 Gy. Full-course radiotherapy with concurrent and adjuvant chemotherapy are commonly used in patients with good status, however abbreviated course of "hypofractionated" radiotherapy with concurrent and adjuvant chemotherapy is proper for elderly patients with bad performance and poor prognosis.

*Chemotherapy* is another approach for treating glioblastoma. Temozolomide (TMZ) is a DNA-alkylating agent that can cross the blood-brain barrier (BBB) and achieve proper concentration in brain. TMZ concomitant to radiotherapy followed by maintenance TMZ treatment has become a standard care for patients with glioblastoma. TMZ leads to DNA strand breaks by adding methylation in DNA residues, such as N<sup>7</sup>-methylguanine, N<sup>3</sup>-methyladenine, and O<sup>6</sup>-methylguanine, if they are not repaired by O<sup>6</sup>-methylguanine-DNA methyl-transferase (MGMT). Thereby the *MGMT* promoter methylation is a good prognostic marker for TMZ treatment. However, TMZ treatment in low-grade glioma has a risk to induce transformation to an aggressive high-grade glioma by induction of hyper-mutation and hyper-methylated status [12]. In addition, an anti-angiogenic agent (anti-VEGFA, bevacizumab), is used in glioblastoma patients. Low dose of bevacizumab together with standard radiotherapy and chemotherapy is utilized to decrease cerebral edema. The combination of bevacizumab with hypofractionated radiotherapy improved overall survival in elderly compared with radiotherapy alone, although this benefit was not observed in primary glioblastoma [13, 14].

*Tumor Treating Fields (TTFields)* is a new care approach in newly diagnosed glioblastoma patients. The mechanism is that it interferes with polar organelles and inhibits cell division by forming electrical fields of 200 Hz, and also induces anti-glioma immune response. Patients that received adjuvant TMZ and TTFields after initial treatment with TMZ/radiotherapy showed longer survival than those treated with TMZ alone [15, 16].

## 1.4 GENOMIC ALTERATIONS IN GLIOBLASTOMA

Current technologies such as large-scale genomic DNA analysis have eluded to the genetic evolution of glioblastoma. According to comparative genomic hybridization (CGH), the gain of chromosome 7p, with the epidermal growth factor receptor (*EGFR*) locus and the loss of chromosome 10q, with the phosphatase and tensin homologue (*PTEN*) locus are the most frequent alterations [17]. Besides this, gene mutations such as *IDH*, *p53*, *PTEN*, and their involved signaling pathways, including RTK/RAS/PI3K, *p53* signaling, and *RB* signaling are critical in gliomagenesis [18] (Figure 2).

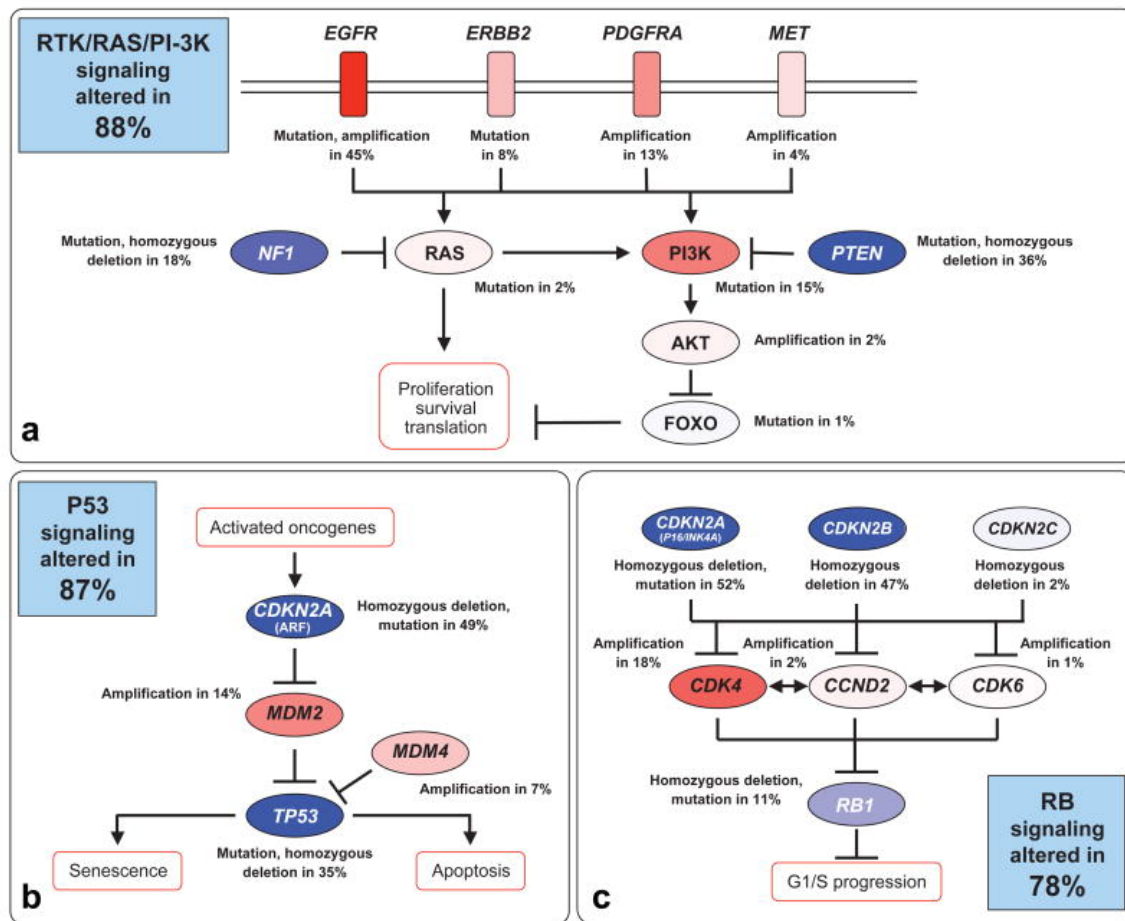


Figure 2. Frequent genetic alterations in three critical signaling pathways. Adapted from [18], with permission.

### *IDH* mutations

The main function of *IDH1* is to convert isocitrate to  $\alpha$ -ketoglutarate ( $\alpha$ -KG), while the co-factor nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) is oxidized to NADPH. However, the mutated *IDH* reduces  $\alpha$ -KG to 2-hydroxyglutarate (2-HG) instead, and produces NADP<sup>+</sup> from NADPH. The increase of 2-HG and reduction of  $\alpha$ -KG can promote tumor development in several ways, such as blockade cell differentiation [19], activation of hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) and its downstream factors [20], and induction of tumor angiogenesis [21]. In glioblastomas, *IDH1/2* mutations can be found in up to 5% of primary

glioblastoma cases, whereas it is present in almost 80% of secondary glioblastoma [22]. *IDH* mutations are also linked to epigenetic alteration of cytosine-phosphate-guanine (CpG) island methylator phenotype (G-CIMP) [23].

#### *p53 mutation*

The ARF-MDM2-p53 pathway has been found deregulated in about 87% of glioblastomas [18]. Common mutations include missense *TP53* mutations, deletion of *CDKN2A/ARF*, and/or amplifications of *MDM2*, and/or *MDM4*. *MDM2* gene is located on chromosome 12q and is amplified in approximately 10-15% of glioblastomas [18]. *TP53* mutations are more common in secondary (65%) than in primary (30%) glioblastomas [24]. p53 is a tumor suppressor and regulates DNA repair, cell senescence, cell death and metabolism [25]. In response to oncogene activation, ARF has the ability to inhibit the E3 ubiquitin ligase protein MDM2 that normally degrades p53, thus causing stabilization of p53. MDM2 is also a downstream target gene of p53 and p53 level is controlled thereby in a feedback loop. Inhibiting MDM2 from binding to p53 causes immediate stabilization of p53. Mutant p53 is unable to induce the *MDM2* gene and mutant p53 may therefore accumulate to higher levels in tumors but this may depend on the mutation in question [26]. Moreover, a subset of p53 mutants can be functional classified as gain-of-function (GOF) mutants, which was reported to attract microglia or monocytes inflammation in glioblastoma and correlated with shorter survival in patients [27].

#### *PTEN mutation*

PTEN is a tumor suppressor gene and locates in chromosome 10q. The main function of PTEN is to negatively regulate the phosphatidylinositol-3-kinase (PI3K)/AKT pathways and control cell growth, metabolism and survival [28]. PTEN inactivation by mutation or deletion occurs in 36% of glioblastoma. The loss of PTEN promotes glioblastoma cells proliferation by increasing AKT/mTOR activity. In the cells with PTEN and wild type p53, they cooperate and enhance the tumor suppressive function of each other. PTEN is able to inhibit the degradation of p53, whereas p53 can activate PTEN by binding to its promoter [29, 30]. Conversely, in the cells expressing wild-type PTEN and mutant p53, PTEN can be said to have a tumor promoting properties. PTEN inhibits the degradation of mutant p53 by inactivation of MDM2 as well as direct stabilization of mutant p53 protein, where inhibition of PTEN suppresses cell proliferation and tumor growth [31].

#### *EGFR amplification*

*EGFR* is located on chromosome 7p. *EGFR* amplification occurs in up to 40% of glioblastomas, and furthermore *EGFR* mutations (*EGFRvIII*) occur in half of tumors with amplified *EGFR*. *EGFRvIII* is a mutated form of *EGFR* with deletions in exons 2 and 7, that is a loss of 267 amino acids in the extracellular domain, and it shows less binding capacity to ligands [32]. In glioblastoma, wild type *EGFR* and *EGFRvIII* are always co-expressed, but *EGFRvIII* is more tumorigenic and activates the PI3K/AKT, RAS, and MAPK pathways in

cells. EGFRvIII and wild type EGFR form autocrine/paracrine loops in glioma cells, where the cells with EGFRvIII supply ligands, such as heparin binding epidermal growth factor (HB-EGF) and transforming growth factor  $\alpha$  (TGF $\alpha$ ), for the proliferation of cells with wild type EGFR [33].

#### *PDGFRA amplification*

Platelet-derived growth factor receptor- $\alpha$  (*PDGFRA*) amplification is found in about 13% of glioblastoma. There are two main types of rearrangements associated with *PDGFRA* amplification. One is a gene fusion with *VEGFR2*, and the other one is an in-frame deletion of exon 8 and 9 (mutated *PDGFRA*). Mutant *PDGFRA* is a recurrent gene rearrangement occurring in 40% of the glioblastomas with *PDGFRA* amplification. Amplified *PDGFRA* has oncogenic properties in glioblastoma with elevated tyrosine kinase activity and transforming potential [34].

#### *TERT promoter mutation*

Telomerase reverse transcriptase gene (*TERT*) locates on chromosome 5p15.33. *TERT* acts to maintain the length of telomeres by using its own RNA. *TERT* promoter (*TERTp*) mutation, where the two most frequent ones are C228T and C250T, activates *TERT*, resulting in elongated telomeres [35]. *TERT* mutations are frequent occurring in 69% of glioblastoma and related with higher risk of glioblastoma [36]. In cancer, increased telomerase expression by for example mutated telomerase promoter, or by an alternative lengthening of telomeres (ALT) promotes cells long-time survival [37].

#### *MGMT hypermethylation*

*MGMT* is located on chromosome 10q26.3, which can remove alkyl adducts from the O6 position of guanine, therefore it has a function in DNA repair. *MGMT* hypermethylation is observed in approximately 50% of GBM and occurs at the cytosine-phosphate-guanine (CpG) island sites in the promoter area of *MGMT* [38]. Hypermethylation of *MGMT* silences the DNA repair function and results in base misrepair during DNA replication. In glioblastoma, patients with *MGMT* methylation have better response to alkylating chemotherapy[39].

## 2. GLIOBLASTOMA HETEROGENEITY

Tumor cell heterogeneity has become a hot topic of discussion in the glioblastoma field, and also in several other tumor types. Tumor cell heterogeneity refers to a variation in phenotypic, epigenetic, or genetic features. Due to the heterogeneity tumor cells bearing different abnormalities are predicted to respond differently to therapies. Therefore understanding of the heterogeneity is a strong prerequisite to advance personalized treatment and improve clinical outcomes. Heterogeneity can be seen at both an intertumoral and intratumoral level. Intratumoral heterogeneity can be considered at several different levels that to some extent overlap including cancer stem cells and differentiated cells, as well as cancer cells with distinct glioblastoma subtype gene expression signatures.

### 2.1 INTERTUMORAL HETEROGENEITY-SUBTYPES

Intertumoral heterogeneity refers to variation between tumors from different patients. Based on gene expression profiling, glioblastomas are classified into three molecular subtypes, namely classical, proneural, and mesenchymal [40, 41]. Each subtype contains gene expression signatures observed in different neural lineages, implying that different subtypes may have be associated with further phenotypic similarities to these lineages.

The classical subtype has 100% frequency in gain of chromosome 7 and loss of chromosome 10. Tumors with the amplification and/or mutation in *EGFR* (*EGFRvIII*), located on chromosome 7, show high enrichment in classical subtype. Co-occurring with *EGFR* amplification, focal *CDKN2A/CDKN2B* deletion, which is located in 9p21.3, happens in 95% of classical subtype. Classical subtype has higher expression of the neural precursor and stem cell marker *NESTIN*, genes in the NOTCH pathway, such as *NOTCH3*, *JAG1*, and *LFNG*, as well as genes in sonic hedgehog signaling (SHH) pathways, such as *SMO*, *GAS1* and *GLI2* [40].

The proneural subtype displays characteristics including *PDGFRA* amplification, *IDH1* and *IDH2* mutations as well as *TP53* mutations. Mutation of *PDGFRA*, or amplification at its gene location in the 4q12 locus happens in all subtypes, but more frequently in proneural cases compared to others, as well as high *PDGFRA* gene expression. *IDH1* mutations (11/12), *TP53* mutations (20/36) and loss of heterozygosity (LOH) (10/15) occur frequently in proneural subtype. Chromosome 7 gain and chromosome 10 loss occur in 54% of proneural cases. In proneural subtype, oligodendrocytic and proneural development genes, such as *PDGFRA*, *OLIG2*, *DLL3*, and *SOX* genes, are highly expressed, indicating its association with oligodendrocyte lineage cells [40].

The mesenchymal subtype is characterized by loss of *NFI*, accompanied by the focal deletion of a region at 17q11.2, and a majority of the samples have low *NFI* gene expression level. It has been shown that among 20 samples with *NFI* mutations, 14 of them belonged to the mesenchymal glioblastoma subtype. Among seven samples that had co-mutations of *NFI* and

*PTEN*, six of them are observed in the mesenchymal subtype. Mesenchymal markers such as *CHI3L1*, *MET*, *TNF*, and several NF- $\kappa$ B pathway genes are higher expressed in this group [40].

However, combined omics results from tumor tissue analyses do not reliably to explain the complex cellular processes occurring within the glioblastoma tumor mass [42]. Some samples don't fall into either of these categories or seem to be composed of a mixture. This suggests that classification into three subtypes was not sufficient. Extensive evidence has recently shown that tumors are composed of many different cell clones, that is intratumoral heterogeneity. In 1987, Nistér et al. established diverse cell lines originating from the U343 glioma tumor and showed they are phenotypically different although they have the same origin [43]. Recently, single cell based approaches have revisited the question of intratumoral heterogeneity in glioblastoma. Collectively, these reports indicate that intratumoral heterogeneity is complex and essential to study further.

## **2.2 INTRATUMORAL HETEROGENEITY-GLIOMA STEM CELLS**

Cancer stem cells (CSCs) is a population of tumor cells that are able to sustain tumorigenesis [44]. During tumor development, CSCs differentiate to various non-CSC cells. CSCs have been assigned to have a high resistance to drug treatment and to cause tumor relapse. Therefore targeting of CSCs is a heavily researched therapeutic strategy. Nevertheless, potential dedifferentiation between a differentiated and a CSCs state, which is regarded as tumor cell plasticity and contributes to intratumoral heterogeneity, may hamper the success of CSCs targeting approaches. Moreover, glioma initiation cells (GICs), or tumor initiation cells (TICs) is a name that in certain contexts are interchangeably used for CSCs, have been reported to contribute to heterogeneity. Segerman et al. have shown that the drug and radiation sensitive GICs have a more proneural signature, while resistant cells have a more mesenchymal signature [45]. Therefore, better understanding of glioma stem cells (GSCs) and GICs would facilitate proper development therapeutic approaches targeting this tumor cell population.

### **2.2.1 Intrinsic modulation of GSCs: genetic and epigenetic modification**

Many transcription factors have been shown important for GSC maintenance. Four transcription factors including POU3F2, SOX2, SALL2 and OLIG2 were identified to reprogram differentiated glioblastoma cells to stem-like tumor progenitor cells (TPCs), where 50% of these cells expressed CD133. Moreover, these factors acquired H3K27 acetylation at their promoters, which sets them in an active state [46]. Forkhead box protein M1 (FOXM1) is also a transcription factor that regulates GSC. FOXM1 binds with the mitotic kinase maternal embryonic leucine zipper kinase (MELK), which functions to phosphorylate and activate FOXM1. The inhibitor of MELK, OTSP116, was shown effective in xenografts and promising therapeutic agent for glioblastoma [47]. FOXM1 also binds with *PDGFA* promoter

and promote PDGFA-STAT3 signaling to maintain the tumorigenicity of GSC [48]. YAP (Yes-associated protein) and MRTF-A (myocardin-related transcription factor A) can co-activate Rho-mediated gene expression, and is required for GSC proliferation and tumorigenicity [49]. GSCs also display robust circadian rhythm for the growth depending on the core clock transcription factors, BMAL1 (Brain and muscle Arnt-like protein1) and CLOCK (Circadian locomotor output cycles kaput). The suppression of BMAL1 and CLOCK induced GSCs cell cycle arrest and increased apoptosis [50].

DNA methylation, as an epigenetic modification, has also been reported as a driver of CSC formation and maintenance. The methylation status depends on the DNA methyltransferases (DNMT1, DNMT3A, DNMT3B) and methylcytosine dioxygenases (the ten-eleven translocation, TET1 and TET2). DNMT3A mutation is the most common mutation that induces DNA methylation and confers self-renewal property of tumor cells [51]. TET proteins oxidize 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). GSCs have been found with global loss of 5mC and 5hmC, accompanied by gain of 5fC and 5caC. Glioblastoma patients with low 5hmC have been reported with shorter survival [52]. EZH2 (Enhancer of Zeste Homolog 2), is a polycomb repressor complex 2 (PRC2) methyltransferase subunit. Phosphorylation of EZH2 has been shown to methylate and activate STAT3 for GSCs maintenance [53]. Moreover, RNA methylation is important for GSCs. N6-methyladenosine (m<sup>6</sup>A) modification is a most prevalent RNA modification, which is catalyzed by methyltransferase-like 3 and 14 (METTL3 or METTL14). Knockdown of METTL3/METTL14 increased GSCs self-renewal and tumorigenesis [54].

Therefore, a diverse set of transcription factors and methylation are involved in the regulation of GSCs. More knowledge on these factors will be helpful for targeting GSCs, for example, inhibition of methylation, such as by using DNMT inhibitor 5-azacytidine, would be effective in eliminating GSCs and get better response in glioblastoma patients.

## **2.2.2 Extrinsic modulation of GSCs: microenvironment**

*The perivascular niche* is important for GSC maintenance. It has been reported that injection of GSCs in the subependymal zone of brain coronal organotypic slices induced a tumor cell response to endothelial niche signaling in the host brain [55]. Interaction between GSCs and endothelial cells (ECs) and/or pericytes has been shown to influence glioblastoma progression. In a PDGF-driven mouse model of glioblastoma, PDGF was able to induce endothelial nitric oxide synthase (eNOS) and activate NOTCH1 signaling to maintain GSC phenotype. Further, PDGF induces cell stemness via inhibitor of differentiation (ID). Inactivation of ID proteins decreases stemness in GSCs. Therefore a PDGF-ID-NO-NOTCH axis plays an important role in GSCs maintenance [56, 57]. The expressed NOTCH1 ligands Delta-like-4 (DLL4) and Jagged-1 (JAG1) in ECs can bind with NOTCH1 in GSCs and promotes tumor angiogenesis and growth [58]. Moreover, ECs secrete TGF $\beta$  and PDGF to

increase stemness genes expression, such as *SOX2*, *OLIG2*, *BIM-1*, and *PROM1* (*CD133*) [59]. TGF $\beta$  from ECs helps GSCs to give rise to pericytes, which in turn protect endothelial cells and promote tumor growth. Removal of pericytes increases the sensitivity towards radiation or chemotherapy treatment and inhibits tumor growth [60]. Furthermore, the surrounding microenvironment increases inducible nitric oxide synthase (iNOS) production in GSC, and enhances sphere formation, tumorigenic potential. High iNOS correlates with bad prognosis [61].

*Extracellular matrix (ECM)*, such as laminin, integrin and cadherin, interact with GSCs. Laminin  $\alpha 2$  and  $\alpha 4$  are expressed in mesenchymal cells, play a role in maintenance of GSCs, and contribute to tumor invasion and recurrence [62]. The laminin receptors, such as integrin  $\alpha v \beta 3$ ,  $\alpha v \beta 5$ ,  $\alpha 5 \beta 1$ ,  $\alpha 3$  and  $\alpha 6$  have been shown expressed in glioblastoma.  $\alpha 5 \beta 1$  is expressed in mesenchymal subtype glioblastoma, and it modulates survival of GSCs by negative regulation of p53 [63]. In addition, cadherin is important in cell-cell interaction and tumor invasion. N-cadherin is expressed in normal stem cells and functions to maintain progenitor state [64]. In glioblastoma, there is an alteration in cadherin expression, with down-regulation of E-cadherin that is associated with poor progression-free survival in glioblastoma, but with no effect on overall survival [65].

*Hypoxic niche* is a hallmark in glioblastoma. GSCs are enriched in hypoxic regions with activation of HIF-1 and HIF-2, which affects GSC self-renewal, proliferation and invasion. Hypoxia induces c-Met expression by HIF-1 $\alpha$ , and enhances cell invasion by activation of hepatocyte growth factor (HGF) [66]. HIF-2 also regulates and promotes GSC phenotypes [67]. HIF-2 $\alpha$  increases the expression of *SOX2*, *KLF4*, *OCT4* and *c-Myc* [68]. Cells that are exposed to long-time hypoxia shift to stem-like state [69]. Hypoxic regions are heterogeneous with different degrees of oxygen tension, which may also contribute to spatial heterogeneity in glioblastoma.

Besides, *immune cells* also contribute to the GSCs maintenance. I will describe it in the fourth part in this thesis.

Above all, GSCs cooperate with surrounding microenvironment to maintain survival and growth. GSCs modification induced by intrinsic genetic and epigenetic changes, as well as by the microenvironment would contribute to the intratumoral heterogeneity in glioblastoma [70].

### **2.2.3 GSCs plasticity**

The bi-directional inter-conversion between stem and non-stem cancer cell populations is a contributing factor for glioblastoma plasticity [71]. In mammalian development, stem cells and progenitor cells can regulate transcription factors, chromatin regulators and associated cellular networks, resulting in forming different cell types. It has been shown that introduction of oncogenes into neural stem cells, progenitors and/or astrocytes in mice brain



generated malignant glioblastoma like tumors, accompanied by increasing expression of stem or progenitor cell markers compared to differentiation markers. This suggests a plasticity between differentiated cells and stem cell phenotype in glioblastoma [72]. Yamanaka et al. have made the stem cell plasticity to reality by generating induced pluripotent stem cells (iPSCs) from murine fibroblasts, where they transduced four transcription factors SOX2, Oct-3/4, c-MYC, and KLF4 in fibroblasts [73]. Sura et al. further showed a core set of four transcription factors that coordinately bind and activate tumor propagating cells (TPCs) specific elements and sufficiently reprogram differentiated glioblastoma cells to induced TPCs [46]. The transition between non-GSCs and GSCs happens in response to microenvironment, such as perivascular niche, ECM, hypoxia and treatment. Bone morphogenetic protein 4 (BMP4) is a protein belonging to the TGF $\beta$  superfamily and important for early differentiation of embryo. Short time exposure of BMP4 on glioma stem cells decreased the tumor propagating capacity [74]. TMZ treatment in differentiated cells induces expression of Sox2, Oct4, CD133 and Nestin in GSCs, and these GSCs are more efficient to form tumor when intracranially implanted in mice [75]. All of this indicates plasticity between GSC and non-GSC phenotypes in glioblastoma, which also is a cause for intratumoral heterogeneity.

## 2.3 INTRATUMORAL HETEROGENEITY-GLIOMA CELLS

### 2.3.1 Intratumoral heterogeneity in glioblastoma

Intratumoral cancer cell heterogeneity in glioblastoma manifests that different clones with distinct genomic or epigenetic alterations, morphologies, and phenotypes coexist within individual tumors, with spatial and temporal distribution patterns [76]. It has been speculated that the intratumoral heterogeneity occurs during tumor evolution. Genetic alterations in receptor tyrosine kinase (RTK) genes, such as amplification of *EGFR*, *MET*, and *PDGFRA*, have been reported existing in the single glioblastoma. According to FISH analysis, these kinases are amplified in different cells in a mutually exclusive fashion, but they share common early genetic mutations, such as homozygous deletion of *CDKN2A* or *TP53* mutation. This indicates these cells are derived from a single precursor cell during tumor evolution. Several amplified RTKs coexisting in the same tumor suggests that combinational therapy, such as gefitinib (EGFR inhibitor) and imatinib (PDGFR inhibitor), is necessary to obtain complete inhibition of the PI3K signaling and suppress tumor growth [77, 78]. Ozawa et al. have shown that the common order of tumor evolution is initial gain of chromosome 7 and loss of chromosome 10, then *CDKN2A* loss or *TP53* mutation, and then alterations in the specific subtypes genes [79].

Sottoriva A et al. has uncovered this heterogeneity in glioblastoma by performing integrated genomic analysis on multiple biopsies taken from the same glioblastoma. They identified the chromosomal gain of *EGFR* and loss of *CDKN2A/B* in the early stage during cancer progression, however gain of *PDGFRA* and loss of *PTEN* were later events. Moreover, they

noticed different subtypes, proneural, classical, and mesenchymal subtypes present in the same tumor by using the Verhaak classifier genes [80]. Proceeding this, Patel et al. performed single cell RNA-sequencing on single cells from the same tumor, and the data showed that the individual glioblastoma is composed of cell subpopulations that have different proneural, classical or mesenchymal subtype signatures [81]. Meyer et al.'s study focused on the functional profiling of different clones in glioblastoma. They found these clones display unique proliferation, differentiation, tumorigenic and drug response abilities. Also therapy resistant clones preexist in the untreated glioblastoma, and express higher genes involved in cell migration (*MET*) and angiogenesis (*WNT17B*, *EGF*) compared to the TMZ sensitive clones [82].

### 2.3.2 Proneural to mesenchymal transition (PMT)

Some cancer cells reversibly transition between different phenotypic states to contribute to tumor growth, named plasticity [83]. Deeper knowledge on the plasticity may lead to new potential targets for glioblastoma treatment and to new ways of confronting resistance. Currently, many studies have reported genetic alterations and signaling mechanisms that results in glioblastoma phenotype plasticity, where proneural to mesenchymal transition (PMT) is most studied. Ozawa et al. have shown *PDGFA* and *PTEN* are major drivers of glioblastoma. *PDGFA* was sufficient in the initiation of proneural-like gliomas, which was enhanced by *PTEN* loss. However, this proneural phenotype could be induced to a mesenchymal subtype by additional *NFI* loss, suggesting mesenchymal subtype evolves from a proneural precursor [79]. Anti-angiogenic therapy (bevacizumab) provokes aggressive treatment resistant clones, which is associated with mesenchymal subtype, indicating that PMT may be an effect of anti-angiogenic therapy [84]. Many signaling pathways such as STAT3 and C/EBP $\beta$ , Hippo pathway TAZ, as well as the NF- $\kappa$ B pathway have been shown to be involved in PMT [85-87].

*STAT3* and *CEBPB*, as well as *bHLH-B2*, *FOSL2*, *ZNF238* and *RUNX1*, are identified as master regulators in mesenchymal gene expression signature [86]. Halliday et al. found that radiation rapidly induces mesenchymal transition via the up-regulation of *STAT3* and *CEBPB* in glioma [88]. The Janus kinase 2 (JAK2) is a STAT3 activator. JAK2-STAT3 has been shown to regulate many cellular processes in glioblastoma. Combination of JAK inhibitor AZD180 with radiation, compared to radiation alone, results in better survival in mice [89].

TAZ is a downstream factor of the Hippo pathway. It is a master regulator in PMT according to Bhat's study, where TAZ expression is lower in proneural glioblastoma and low-grade gliomas, but higher in mesenchymal, and loss of TAZ in mesenchymal glioma stem cells or overexpression in proneural glioma stem cells affects mesenchymal gene expression signature. Chromatin immunoprecipitation (ChIP) showed TAZ is directly recruited to the promoter of MES genes in a complex with TEAD (transcriptional enhanced associated domain). The co-expression of TAZ and PDGFB induced a mesenchymal tumor [87].

NF- $\kappa$ B pathway is also involved in PMT. Bhat et al, reported a NF- $\kappa$ B dependent mesenchymal transition with an associated enrichment of CD44 expressing cells and a gain of radio resistance, where macrophage/microglia was the potential microenvironment regulator. In support of this, immunohistochemistry analysis showed positive NF- $\kappa$ B activation signal in MES regions and close to macrophage/microglia infiltration zone [90]. In Yin's study, they showed an increased expression of transglutaminase 2 (TGM2) in glioblastoma perinecrotic region, induced by macrophage/microglia-derived cytokines via NF- $\kappa$ B signaling. TGM2 is an enzyme that catalyzes  $\text{Ca}^{2+}$ -dependent protein crosslinking and increases GSCs differentiation to MES subtype by upregulation of TAZ, CEBPB and/or STAT3 [91]. CXCL1 is a chemokine and highly expressed in glioblastoma. It has been shown that overexpression of CXCL1 in glioma cells induced mesenchymal transition via activation NF- $\kappa$ B pathway and increased radioresistance [92].

Besides PMT, transition exists between other phenotypes. Olig2 is an early marker for oligodendroglial lineage progenitors correlated with proneural phenotype. The ablation of OLIG2 causes a phenotype shift from proneural towards classical phenotype with activation of EGFR signaling, which reduces tumor initiation and growth and makes gliomas sensitive to EGFR inhibition [93].

### **2.3.3 Glioblastoma plasticity and tumor recurrence**

Tumor recurrence following treatment is one of the causes for cancer mortality. The recurrent tumors are usually not sensitive to the original therapies since the genomic alterations are distinct from those in the initial tumor. There are two theories to explain the role of genomic alterations. First, based on the intratumoral heterogeneity, treatment sensitive clones would be removed as a result of the treatment, and then the remaining resistant clones grow up and form new tumors. In another theory, the cancer cell bulk will be killed, except for the cancer stem cells (CSC) since they display more drug-resistance. Then CSCs give rise to new cells and to regrowth of the tumor.

Recently, multiregional sequencing efforts have been made by profiling multiple regions in the primary and recurrent tumors to elucidate the evolution of tumor recurrence. From that, both linear evolution (the recurred tumor share majority of mutations with initial tumor) and divergent evolution (the recurred tumor share minority of mutations with initial tumor) were identified. Johnson et al. have sequenced the exosomes from both initial and recurrent tumor resections from the same patient, and noticed that many mutations present in the initial tumor, such as *TP53*, *ATRX*, *SMARCA4*, and *BRAF*, were not detected in the recurrent tumor. Moreover, recurrent tumors after TMZ treatment show inactivation of the DNA mismatch repair (MMR) pathway, deregulation of RB pathway such as *RBI* and *CDKN2A* mutations to inhibit cell cycle arrest, and activation of Akt-mTOR signaling, such as *PIK3CA* and *PTEN* mutations to active Akt hyperactivation [94]. Kim et al. further showed that glioblastoma recurrence at a distant location had more divergent drivers alterations than recurrence in the

same location as the initial glioblastoma, indicating that re-biopsy and re-profiling are necessary for the distally recurring tumor. In addition, primary glioblastoma with wild-type *IDH1* rarely develop hypermutations after TMZ treatment in contrast to *IDH1*-mutant gliomas [95]. In summary, these studies provide explanations for the ineffective treatment in the recurred tumors, and more insights into the evolutionary process are critical for improving patient care.

### 3. CELL-TO-CELL COMMUNICATION IN THE HETEROGENEOUS GLIOBLASTOMA

Cancer progression depends on cooperation between tumor cells and other neighboring non-tumor cells in the microenvironment. The distinct cancer cell subsets may also cooperate with each other and contribute to the malignant phenotype [96, 97]. Quite a few studies have focused on the communication between glioblastoma cells and surrounding immune and vascular cells, whereas there are limited reports on interactions between different glioblastoma cells. Davis et al. have constructed a model for clonal interactions and demonstrated that clonal heterogeneity is an intrinsic property and influences each other, for example cancer clones cooperate and protect slow growing clones. Moreover, they noticed clones that are drug sensitive can obtain resistance when cooperating with the drug-resistant clones [98]. This indicates more understanding on cell-to-cell interactions will provide clues to further therapeutic targets. Cell-to-cell interaction happens in many ways. Here I will introduce secreted factors, direct cell-to-cell contacts, gap junctions, and extracellular vesicles.

#### 3.1 SECRETED FACTORS

Secreted factors are important for the cells inter-communication, such as interleukins (ILs), Wnt ligand and HGF, and so on. The cell clone with *EGFRvIII* communicates and promotes the proliferation of surrounding wild type *EGFR* tumor cells by secretion of IL6 and Leukemia Inhibitory Factor (LIF) [99]. IL6 could activate NF- $\kappa$ B and induce the expression of prosurvival protein survivin (BIRC5). Moreover, IL6 was found to change the sensitivity to EGFR tyrosine kinase inhibitors (TKIs), an effect mediated by bromodomain protein 4 (BRD4), suggesting that the combination treatment of TKIs and inhibition of BRD4 would benefit glioblastoma patients [100].

WNT ligands are secreted proteins and WNT pathway has been shown involved in cell stemness, invasion and angiogenesis in glioblastoma. Norrin, which is encoded by Norrin disease protein (*NDP*), is an atypical Wnt ligand that can bind with Frizzled receptor 4 (FZD) and activate Wnt signaling. Norrin has been shown to have suppressive and promoting functions in ASCL1<sup>lo</sup> and ASCL1<sup>hi</sup> GSCs, with an activation of Wnt signaling and Notch signaling mechanisms, respectively. ASCL1 (Achaete-scute homolog) is a basic helix-loop-helix transcription factor and used a proneural factor, and high and low expression cells exhibit different differentiation and invasion dynamics [101]. Protocadherin FAT1, a tumor suppressor-related gene that encode a cadherin-like protein, can antagonize WNT signaling by binding with  $\beta$ -catenin and inhibiting its nuclear translocation. The homozygous deletion of FAT1 has been found in 20% of glioblastoma and leads to aberrant Wnt activation during tumorigenesis [102].

HGF (Hepatocyte growth factor)/MET (The mesenchymal epithelial transition) signaling is also associated with poor prognosis in glioblastoma patients, with the downstream effects

such as MAPK, PI3K/AKT, STAT3, and NF- $\kappa$ B, resulting in cell proliferative, invasive, and survival capacity [103]. It has also been shown as a mechanism for the resistance to EGFR or VEGFR inhibitors during the tumor treatment [104, 105]. Moreover, it was shown that inhibition of c-MET decreased nuclear translocation of  $\beta$ -catenin, indicating there are overlaps between c-Met and Wnt- $\beta$  catenin in glioblastoma [106].

### 3.2 CELL-TO-CELL CONTACT

Direct cell-to-cell contact is another important mechanism in the communication between cancer cells. Notch signaling mediates direct cell-to-cell interaction via binding of NOTCH ligands (Jagged-1 and 2 and Delta-like 1,3,4) on one cell to NOTCH receptors (Notch 1-4) on the adjacent cell to induce the enzymatic cleavages of Notch intracellular domain (NICD). Then NICD travels to the nucleus and leads the upregulation of Hes and Hey family members. NOTCH1 has been reported to crosstalk with other pathways, such as NF- $\kappa$ B factors (P50, P52, P65, RelB, and cRel). NOTCH1 promotes glioblastoma progression through the binding of NICD to P65, and affects cell proliferation and apoptosis [107].

Hippo-YAP pathway has also been shown to control cell proliferation. Some upstream modules, such as cell-cell contact, cell-matrix contact, and also some extracellular growth factors, can regulate hippo pathway. The activation of Hippo leads to activation of Lats1/2, which induces phosphorylation and degradation of YAP/TAZ in cytoplasm, resulting in suppression of cell proliferation [108]. *YAP* and *TAZ* are downstream transcriptional coactivators in the Hippo pathway, and have been shown to regulate EMT and tumor survival together with KRAS [109].

Bradykinin and its receptor B1R binding have also been reported to increase glioma cell migration via PI3K/Akt pathway. Bradykinin (BK) is a peptide that promotes inflammation and also modulates BBB permeability [110]. In the three-dimensional (spheroid) co-culture model of glioblastoma cells U87 and bone marrow derived mesenchymal stem cells (BM-MSCs), U-87 expressed higher B1R and became more invasive upon stimulation of B1R agonist des-Arg<sup>9</sup>-bradykinin, compared to 2D mono-culture, indicating the importance of cell-to-cell contact during tumor progression [111].

### 3.3 GAP JUNCTIONS

Gap junctions connect glioma cells and form intercellular networks enabling intercellular communications over long distances. Gap junctions are composed of membrane proteins called connexins, that function to transmit intercellular calcium waves (ICW) and bidirectional exchange of ions, microRNAs or other small molecules between cells. Disruption of gap junctions will isolate cells from this network and become more sensitive to the treatment.

In 1997, Chosh and Singh reported the role of gap junctional channels in glioma cells communication, where they observed decreased proliferation and increased differentiation of C6 glioma cells after exposure to dibutyryl cyclic AMP (dbcAMP). Moreover, the differentiated cells had more fine processes, which increased the frequency of cell-to-cell communication more than the body-to-body contact in undifferentiated cells [112]. Another report stated that EGF stimulation increased migration of glioma cells, accompanied by a decrease of connexin 43 (Cx43) level to detach the glioma cells from neighboring cells [113]. The first gap junction inhibitor, INI-0602, which can cross the blood-brain-barrier, renders glioma cells more sensitive to TMZ treatment through activation of JNK pathway [114]. Furthermore, gap junction is involved in transferring microRNA between cells. For example, enhanced gap junctions by retinoic acid treatment helped transferring microRNA-124-3p to adjacent glioma cells, which augmented the anti-proliferative function of miR-124-3p in glioma cells, whereas inhibition of gap junction eliminated this function [115].

### 3.4 EXTRACELLULAR VESICLES

The extracellular vesicles (EVs), which are carriers of oncogenic drivers, microRNA and proteins, are found differentially expressed in glioblastomas of distinct subtypes, and also mediate cell-to-cell communication. EVs are accessible in biofluids, and are suggested as biomarkers in glioblastoma and may influence stem cell hierarchy and tumor microenvironment [116]. EVs in glioblastoma are involved in tumor cell proliferation, invasion, angiogenesis, and treatment resistance [117].

EVs from glioblastoma cells have been reported to induce the transformation of NSCs to tumor-promoting cells, with increased tumor proliferation and migration capacity [118]. *EGFRvIII*-transformed glioma cells, relative to *EGFRvIII* negative cells, are enriched with focal adhesion related proteins and pro-invasive proteins (CD44, BSG, CD151). These cells can deliver the oncogenic property to neighboring glioma cells [119]. The EVs from glioblastoma cells also mediate delivery of microRNA and non-coding RNAs that are conserved in cell cycle, PI3K/Akt and P53 pathways, such as miRNA-21. MiRNA-21 has been found enriched in the cerebrospinal fluid (CSF) from recurrent glioma patients, and is positively correlated with brain metastasis and tumor recurrence [120, 121]. EVs produced in proneural and mesenchymal GSCs are different. EVs from proneural GSCs have less exosomal markers, whereas EVs from mesenchymal GSCs express more canonical CD9, CD63 and CD81 tetraspanins, and are more efficiently internalized by endothelial cells to stimulate endothelial cell growth [122]. Furthermore, EVs play a role during drug treatment. It has been shown that resistant tumor cells can secrete apoptotic extracellular vesicles (apoEVs), which contain spliceosome components, such as RBM11, to alter RNA splicing of *MDM4* and *Cyclin D1* in the surrounding recipient cells, and rendering them more proliferative and oncogenic [123]. Ionizing radiation also induces export of miR-603 through EVs, resulting in suppressing expression of insulin-like growth factor (IGF), IGF1R and MGMT, and acquiring radiation resistance in GSCs [124].

In summary, the direct and indirect communication between cancer cell and cancer cell are important for the whole tumor growth and treatment resistance. Since glioma cells represent different gene expression subtype signatures even in the individual glioblastoma, their crosstalk signaling between each other would be more interesting to investigate. Thereby, a proper glioblastoma cell culture models needs to identify the complexity of the intratumoral heterogeneity.



## 4. MICROENVIRONMENT IN GLIOBLASTOMA

Glioblastoma is an intratumorally heterogeneous tumor that contains not only tumor cells but also non-tumor cells, such as immune cells, astrocytes, and vascular cells, constituting the tumor microenvironment. The tumor microenvironment plays an important role in glioblastoma pathogenesis and progression.

### 4.1 IMMUNE CELLS

Immune cells, including macrophages, dendritic cells, and T cells, perform a defense function in normal physiology and in the early stages of tumor development. However, during tumor progression, the tumor cells produce factors that subvert immune cells to a more tumor protective phenotype, that is to protect the tumor from various immunosurveillance mechanisms [125].

#### 4.1.1 Tumor-associated macrophages/microglia (TAM)

Tumor-associated macrophages/microglia (TAMs) represent the majority of the immune cell population that accounts for an estimated 40% of the tumor mass in glioblastomas [126, 127]. TAMs are composed of bone marrow derived monocytes (BMDMs) and resident-brain microglia cells. BMDMs come from monocytes in the blood and are replenished from blood monocytes. During tumor progression, the blood brain barrier is interrupted and monocytes are then attracted to the tumor by monocytes chemo-attractant proteins (MCPs), such as CCL2, CXCL12, CX3CL1, and glial cell-derived neurotrophic factor (GDNF), and differentiated into macrophages [127]. Microglia are dispersed throughout the brain. It was reported to have developed from immature yolk sac runt-related transcription factor 1 (Runx1)-positive progenitors, and responsible for pathogen clearance and synaptic processes maintenance [128, 129]. During gliomagenesis, microglia are recruited to and by glioblastoma cells and establish a feed-forward cellular circuit to drive tumor growth [127]. Microglia is not replenished from the mononuclear hematopoiesis, and the maintenance depends on self-renewal of resident cells in CNS [129]. In recent years, there have studies on how to distinguish BMDMs and microglia. Trans-membrane protein 119 (*TMEM119*) was shown expressed at a high level on microglia [130], and *ITGA4* /CD49D was identified as a marker for BMDMs [131].

TAMs play an important role in glioblastoma progression and invasion, presumably through several mechanisms. TAMs promote the proliferation and invasion of glioblastoma cells by secreted factors, such as IL1 $\beta$ , TGF $\beta$ 1, MMP2, MMP9 and CCL5 [127, 132-134]. TAMs suppress T-cell activation, and induce T cells producing less pro-inflammatory cytokines and express low level of CD80, CD86 and CD40 [135]. Moreover, TAMs affect angiogenesis and are associated with resistance to anti-angiogenic therapy, such as by bevacizumab [136].

Targeting TAMs is a promising emerging experimental therapy for glioblastoma. Colony

stimulating factor (CSF) is a factor that is important for macrophage growth and survival. Hence inhibition of colony stimulating factor-1 receptor (CSF1R) has been tested in many studies and it reduces glioblastoma growth and prolongs overall survival. Meanwhile, CSF1R inhibition induces glioblastoma cells to secrete granulocyte-macrophage CSF (GM-CSF) and interferon- $\gamma$  (IFN- $\gamma$ ), which increase the phagocytic function in macrophages, and enhance their efficiency in decreasing tumor [137]. Although treatment by CSF1R inhibition prolongs overall survival, tumors recur in more than 50% of mice via activation of IGF1/PI3K pathways. Together with blockade of IGF1/PI3K pathways, CSF1R inhibition significantly prolongs overall survival in recurrent tumors [138].

#### **4.1.2 T lymphoid cells**

The function of T cells in glioblastoma attracts more attention with the development of immunotherapy. Previous studies have shown that both CD8<sup>+</sup> T effector cells and suppressor cells (Treg) exist in glioblastoma [139]. T cells stay in an exhaustion status in tumors, which is mediated by the transcription factor nuclear factor of activated T cells (NFAT). NFAT synergizes with activator protein 1 (AP1) and promotes T cell activation, however in the absence of AP-1, T cells go to an exhaustion status by binding other factors [140]. The activation of “Immune checkpoint” protein is another cause of T cells exhaustion, such as Programmed Death 1 (PD-1), cytotoxic T lymphocytes associated molecule 4 (CTLA4), T cell immunoglobulin 3 (TIM3), and Lymphocyte activating 3 (LAG3), which inhibit T cell proliferation after binding with their ligands. Moreover, the expression of PD-1 or CTLA4 on T cells affects metabolism by decreasing Glucose transporter 1 (GLUT1), an important molecule for glucose uptake. Hence T cells use more glycolysis and produce less ATP, further leading to T cells exhaustion [141]. Studies have shown that the combination of PD-1 and TIM3 antibodies reduces tumor progression in the GL261 glioma model [142]. In another study it was found that the Programmed Death-Ligand 1 (PD-L1), the ligand of PD-1, is increased in mesenchymal glioblastomas [143], and as we know that higher PD-L1 is related with worse outcomes [144]. Importantly, treatment with PD-1 or PD-L1 inhibitors demonstrates significant survival advantages in preclinical studies [145, 146].

Treg is another population of lymphoid cells that is increased in glioblastoma. Treg has a function to suppress T-cell antigen specific response, and the amount of Treg is inversely correlated with patient survival in glioblastoma. Conditioned media from glioblastoma cells increases Treg cells expansion, for example CCL2 produced by macrophages and microglia, and CCL22 secreted by glioma cells are critical for Treg recruitment in the tumor environment [147, 148]. Indoleamine 2,3-dioxygenase (IDO) is an enzyme that is able to inhibit T cells proliferation and induce Treg cells, shows high expression in glioblastoma [149]. Combinational treatment of IDO inhibition with TMZ prolonged survival in a murine glioma model [150]. Moreover, IL2-STAT3 signaling activation contributes to Treg cells expansion. Inhibition of STAT3 by inhibitor STX-0119 decreases Treg prevalence and

increases tumor-infiltrating-T cells in TMZ resistant glioblastoma cells [151].

#### **4.1.3 Dendritic cells (DCs)**

Many immunotherapies have focused on activating CD8<sup>+</sup>T cells by CAR-T or checkpoint inhibitors, however these therapies may be less effective based on the low level of tumor-infiltrating lymphocytes (TILs). DCs therapy may have advantages in the low immunogenic tumors. DCs are one type of antigen-processing cells (APCs), which can process or present antigens to T cells or B cells to induce immune response. Many factors from the tumor cells or tumor microenvironment have been found to suppress DCs activation, such as VEGF, TGF $\beta$ , IL10 and CSF1 [152]. In glioblastoma, NF-E2-related factor 2 (NRF2) was found to be highly expressed in DCs, which is a transcription factor that regulates cellular defense against oxidative stress, and represses inflammation. Conditioned media from glioblastoma cells induced NRF2 expression in DCs and suppressed DCs maturation. In addition, NRF2 was reported to suppress the function of macrophages and facilitated immune escape [153, 154].

Dendritic cell vaccine (DCV) is a DC-based immunotherapy that induces anti-tumor T cell response. DCV has been studied in some cancers such as melanoma with promising results [155]. In the 90s, DCVs have also been tested in N32 and 9L rat glioma cell lines. Both cell lines showed effective response in cytotoxic CD8<sup>+</sup>T cell immunity. Toll-like receptor (TLR) agonists, such as imiquimod, could enhance DCs activation and T-cell immune responses. Combination treatment comprised of DCV and TLR agonists has been reported as a safe and useful adjuvant therapy to the traditional radiotherapy and chemotherapy in newly diagnosed and recurrent glioblastoma patients, with prolonged median survival of 31.4 months [156]. DCV can also induce a shift from Treg cells to Th1/Th17 cells. Together with immunogenic cell death (ICD) that is able to induce Th1 immunity, DCV has generated better immune response in preclinical glioma models [157].

#### **4.2 TUMOR-ASSOCIATED ASTROCYTES (TAA)**

Astrocytes are glial cells that account for 50% of the brain cells. They are important in many physiological processes. Astrocytes are components in the structure of the blood-brain-barrier (BBB), where the end feet of astrocytes bind tightly with endothelial pericytes to maintain the vasculature [158]. During CNS damage, the induced astrogliosis supports tissue repair, where astrocytes alter their properties and become known as reactive astrocytes, with upregulation of the astrocytes-specific intermediate filament protein glial fibrillary acidic protein (GFAP), and of some growth factors and inflammatory factors such as brain derived neurotrophic factor (BDNF), CCL2 and IL6 [159]. In glioblastoma, astrocytes are involved in promoting tumor cell proliferation, invasion, and affecting treatment sensitivity [160]. When co-culturing astrocytes and glioma cells, astrocytes promote glioma cells proliferation and decrease chemotherapy-induced apoptosis [161, 162]. Glioblastoma cells produce receptor

activator of NF- $\kappa$ B ligand (RANKL) to activate NF- $\kappa$ B pathway in astrocytes, and induce astrocytes to become tumor-associated astrocytes (TAAs). Then TAAs secrete TGF $\beta$  to further promote glioblastoma cells proliferation and invasion [163]. Other factors from reactive astrocytes also support glioblastoma cells, such as growth differentiation factor 15 (GDF-15), which belongs to the TGF $\beta$  superfamily. GDF-15 is abundant in glioblastoma patients' cerebrospinal fluid and correlated with shorter survival. The overexpression of GDF-15 promotes glioblastoma growth [164].

Furthermore, reactive astrocytes also play a role for brain metastasis from different primary tumor sources. Active STAT3 in reactive astrocytes correlated with poor patients' survival. Blocking of STAT3 reduced experimental brain metastasis of other tumors [165]. In addition, gap junctions are shown functional between astrocytes and in metastatic brain tumor cells. Connexin 43 is as a component of gap junctions which transfers the 2'3'-cyclic GMP-AMP second messenger (cGAMP) from tumor cells to TAAs, thus induces TAAs to secrete TNF and IFN $\alpha$ , which in turn activate STAT1 and NF- $\kappa$ B pathways in the metastasis tumor cells and induce their proliferation, invasion and resistance to chemotherapy [166]. Therefore, the combination of chemotherapies with gap junction or astrocyte inhibitors should be considered in further therapies for glioblastoma or brain metastasis from other tumors.

#### **4.3 VASCULATURE IN GLIOBLASTOMA**

The blood-brain-barrier (BBB) is an important structure in CNS to maintain the normal function. BBB is composed of endothelial cells, pericytes and astrocytic endfeet. Astrocytic endfeet have function to maintain the endothelial tight junctions and release vasoactive molecules to regulate vascular tone. In glioblastoma, the tumor cells use the perivascular space to invade and displace astrocytes, resulting in disruption of BBB integrity and permeability, which is named blood-tumor-barrier (BTB) [167]. BTB is characterized by aberrant distribution of pericytes and loss of astrocytic endfeet and neuronal connections. There are more immune cells in glioblastoma indicating the enhanced permeability of BTB to immune cells.

Increased blood vessel formation is a striking feature of glioblastoma [168]. Interaction between GSCs and vasculature is bi-directional. GSCs promote angiogenesis by secretion of VEGF or CXCL12 and support GSCs growth [169]. Endothelial cells could be differentiated from GSCs via NOTCH1-signaling. Blocking of NOTCH1 pathway by  $\gamma$ -secretase inhibitor or shRNA decreased the transition from GSCs to endothelial progenitors, whereas anti-VEGF treatment inhibited maturation of endothelial progenitors to endothelium [170]. The GSCs differentiation to endothelial cells was further reported to occur via Tie2 signaling. Tie2 is a tyrosine kinase receptor that is mainly expressed in endothelium. After ionizing radiation treatment, GSCs were shown to generate more vessels, which was inhibited by using Tie2 kinase inhibitor [171]. GSCs also contribute to pericytes formation to support vessel functions. GSCs are recruited to endothelial cells via CXCL12/CXCR4 signaling, where

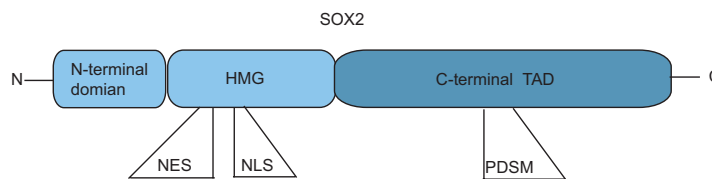
GSCs can differentiate to pericytes via the induced expression of  $\alpha$ -SMA via TGF $\beta$  signaling [60].

On the other hand, the perivascular niche (PVN) supports glioma cells and increases radiation resistance. Osteopontin, a CD44 ligand, promotes cancer stem cell phenotypes and aggressiveness via activation of CD44 signaling [172]. Endothelial NOS (eNOS) is highly expressed in tumor vascular endothelium, which has been shown to activate NOTCH-signaling in PDGF-induced gliomas, and increase neurosphere forming capacity as well as tumorigenic capacity [56]. In co-culture of endothelial cells with glioblastoma cells, IL8 was shown to be secreted by endothelial cells, and promote glioblastoma growth and invasion by increasing CSCs frequency [173].

Above all, immune cells (macrophages, T cells and dendritic cells), tumor associated astrocytes and vasculature in the glioblastoma microenvironment have supportive function in the GSCs maintenance and tumor progression, meanwhile GSCs and glioma cells can induce their formation and infiltration, indicating the interaction between glioma cells and surrounding non-neoplastic cells can provide clues to new therapeutic targets.

## 5. SOX2 IN NORMAL DEVELOPMENT AND CANCER

SOX2 is short for SRY (sex determining region Y)-box 2. Human *SOX2* locates on chromosome 3q26.3-27 and encodes a protein of 317 amino acids. SOX2 is a transcription factor. In the N-terminus, it contains an HMG (high-mobility-group) domain that binds with a specific DNA sequence, the HMG domain is containing conserved functional elements including a nuclear export signal (NES) and a nuclear localization signal (NLS). The C-terminus harbors the transactivation domain (TAD), and contains a phosphorylation-dependent SUMOylation motif (PDSM). SOX2 plays a critical role in normal development and functions also in cancer [174].



*Figure 3. Schematic diagram for structure of SOX2.*

### 5.1 SOX2 IN NORMAL DEVELOPMENT

SOX2 is critical during the early embryonic development and is increasingly expressed from the 2-cell stage to the blastocyst stage. Absence of SOX2 causes embryonic lethality [175]. There are three germ layers during embryonic development, eventually giving rise to different tissue types in the body, which consist of endoderm (inner layer, develops to gastrointestinal tract, lung, liver, pancreas et.al), ectoderm (outer layer, develops to epidermis, hair, and also CNS, PNS et.al) and mesoderm (middle layer, develops to heart, muscle system, urogenital system et.al). SOX2 is able to induce axial stem cells to develop into central nervous system by activation of N1 enhancer, however, T-box Protein 6 (TBX6), which inactivates N1 enhancer and inhibits SOX2 expression, results in driving axial stem cells to mesoderm [176]. SOX2 is highly expressed in neural stem cells, but decreases during the differentiation into neurons and astrocytes. A deficiency of SOX2 caused failure in differentiation to mature GABAergic neurons [177]. Moreover, SOX2 is also involved in endoderm differentiation, such as the formation of trachea [178], stomach and esophagus [179].

SOX2 is highly expressed in embryonic neural progenitor cells (NPCs), but is also expressed in adult neural stem cells (NSCs). Together with OCT4 and NANOG, SOX2 has been shown to be essential for the propagation of undifferentiated NSCs in early development, where these factors collaborate and form regulatory circuits with auto-regulatory and feed-forwards loops to maintain the pluripotency and self-renewal function [180]. SOX2 is one of the Yamanaka factors (SOX2, KLF4, OCT4, c-MYC). Overexpression of Yamanaka factors in somatic fibroblast cells reprograms them to induced-pluripotent stem (iPS) cells [73]. SOX2 also promotes oligodendrocyte differentiation by inhibition of miR145, which targets some

pro-differentiation genes. Loss of SOX2 prevents terminal differentiation of oligodendrocytes but does not affect OPCs [181]. Since SOX2 marks adult stem cells in several epithelial tissues and promotes the generation of many types of cells to maintain tissue homeostasis, ablation of SOX2 has been shown to destroy the tissue homeostasis and lead to lethality [182]. Furthermore, SOX2 has been found subject to age-related decline of expression in many organs. For examples, the expression of SOX2 in peripheral blood mononuclear cells in elderly is less than in younger people, and its expression is inversely correlated with *p16<sup>Ink4a</sup>*. Therefore, SOX2 is also suggested as a biomarker for aging [183].

## 5.2 SOX2 AND CANCER STEM CELLS

CSCs is a small population within the tumor bulk, with stem-like properties, high treatment resistance, and has a function in maintenance of tumor growth and tumor relapse after initial treatment [184]. CSCs have been shown important in the recurrence of many cancers, which eventually survive after chemotherapy and/or radiotherapy, differentiate to daughter cells and reconstruct the tumor, hence an efficient strategy to eradicate CSCs would be useful for cancer therapy.

SOX2 has been reported as a key molecule that drives CSCs in many cancers. In skin squamous-cell carcinoma, SOX2<sup>+</sup> cells are enriched in tumor-propagating cells with stronger tumorigenicity upon serial transplantation, especially of SOX2<sup>+</sup>CD34<sup>+</sup> cells. Conditional deletion of SOX2 in the skin carcinoma leads to tumor regression [185]. In bladder, SOX2 is absent in normal urothelial cells, but it can be found in pre-neoplastic bladder cancer. Cells with SOX2 expression also express other bladder cancer markers, such as keratin14 (KRT14) and CD44v6 [186]. SOX2 has also been shown associated with CSC properties in colorectal cancer, with expression of CD24 and CD44 [187]. In lung squamous cell carcinoma (LSCC), SOX2 has been reported to cooperate with protein kinase C  $\alpha$  (PRKC $\alpha$ ), both of them are oncogenes co-amplified in chromosomal region 3q26. SOX2 is phosphorylated by PRKC $\alpha$  and PRKC $\alpha$ -SOX2 signaling induces activation of Hedgehog signaling to maintain tumorigenic phenotype [188]. SOX2 can also antagonize Hippo pathway to maintain stemness in osteosarcoma [189]. Although SOX2 has a tumor promoting function in many cancers, it has been shown with an opposing effect in gastric cancer, where SOX2 expression is deregulated in cancer compared to normal tissue, due to hypermethylation of *SOX2*. SOX2 exhibits an anti-proliferative and pro-apoptotic function via upregulation of PTEN and AKT dephosphorylation [190]. Above all, SOX2 is correlated with the maintenance of CSC properties and tumor progression in many different tumors.

## 5.3 THE DOWNSTREAM SIGNALING TO SOX2 IN GLIOBLASTOMA

SOX2 has been shown to positively correlate with the glioma malignancy grade, and to be higher expressed in the hyper-proliferative areas. Suppression of SOX2 in glioblastoma tumor-initiating cells (TICs) decreases tumorigenicity [191]. According to several

publications, SOX2 cooperates with many genes and functions in stemness maintenance. Hippo pathway plays a tumor suppressive role in cancer, where two Hippo activators, NF2 and WWC1 (Kibra), exhibit an inhibitory function on the tumor-promoting factor YAP1. In osteosarcoma and glioblastoma, SOX2 was shown to interfere with the Hippo pathways by directly binding to NF2 and WWC1 and reducing their expression, leading to an increase of YAP to maintain CSCs and promote tumorigenesis. This suggests that targeting SOX2-Hippo-YAP would be a therapeutic strategy in these tumors [189]. FOXG1 is a member of the forkhead box family of TFs, and it is one of the most overexpressed genes in glioblastoma-derived tumor spheres. FOXG1 and SOX2 have been shown in a cooperation way to restrict astrocyte differentiation and induce dedifferentiation to stem-like state by targeting cell cycle and epigenetic transcription factors, such as *FOXO3*, *DNMT1*, *DNMT3B* and *TET3*. The combination of Foxo3 loss and DNA methylation inhibitor 5-azacytidin was found to increase astrocytes dedifferentiation [192]. MiRNAs play a role during SOX2-induced reprogramming and dedifferentiation. MiR-486-5p, as a SOX2-induced miRNA, is recently reported to relate with SOX2 expression and stem cell phenotype in glioblastoma. MiR-486-5p can target the tumor suppressor genes *PTEN* and *FoxO1*, indicating SOX2-miR-486-5p axis plays a role to maintain the survival of glioblastoma stem cells. It has been shown that the delivery of miR-486-5p antagomirs in mice xenografts reduced tumor size and also enhanced the response to ionizing radiation [193].

#### 5.4 THE UPSTREAM SIGNALING TO SOX2 IN GLIOBLASTOMA

Many signaling pathways have been reported to maintain the SOX2 expression. SOX2 can be regulated by other genes and to keep cell stemness. GSCs prefer to reside in the perivascular region and get new nutrients for the self-renewal. They also locate along the white matter tracts. It has been reported that early invasive growth of GSCs along white matter tracts contributes to the poor outcome of glioblastoma treatment. The nerve fibers in white matter tracts secrete the Notch ligand, Jagged1, which binds to Notch1 receptor in the CD133<sup>+</sup>Notch1<sup>+</sup> GSCs, and induce SOX9 and SOX2 expression. In a positive feedback, SOX2 inhibits *Notch1* methylation to increase Notch1 expression. Therefore, a NOTCH1-SOX9-SOX2 positive-feedback loop in white matter tracts contributes to maintain GSCs [194]. A TGFβ-SOX4-SOX2 pathway also plays a role in maintaining GSCs. TGFβ directly increases *SOX4*, and *SOX4* associates with the *SOX2* promoter region and promotes *SOX2* expression. An inhibitor of TGFβ deprived of their tumorigenicity, which inhibition could be attenuated by transduction of SOX2 and SOX4. Meanwhile, TGFβ inhibition had a less lethal potency in an intracranial transplantation assay in glioblastoma, indicating that disruption of TGFβ-SOX4-SOX2 pathway would be a potential strategy against glioblastoma [195]. Osteopontin (OPN) is a secreted protein and can activate CD44 signaling in glioblastoma cells to acquire a stem cell phenotype. Targeting OPN in glioblastoma-initiating-cells was shown to decrease the expression of stemness transcription factors, such as SOX2, Nanog and Oct3/4, and abrogated tumorigenic potential in a xenograft mouse model [172, 196]. Wnt/β-



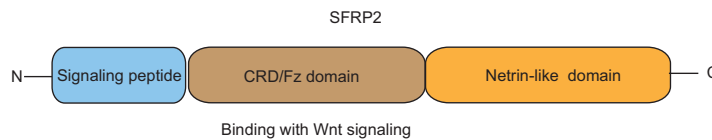
catenin signaling is also connected with CSCs in glioblastoma. Inhibition of Wnt/ $\beta$ -catenin signaling increased TMZ sensitivity, and decreased stem cell markers such as CD133, Nestin and SOX2 [197]. CSC features also exist in circulating tumor cells (CTCs) in glioblastoma, exhibiting SOX2/ETn (early transposon promoter) transcriptional activation and expressing stemness markers such as SOX2, OCT4, and NANOG. CTCs are chemotherapy resistant, and have stronger tumorigenicity with activation of Wnt pathway [198]. SOX2 could be phosphorylated and stabilized by G1 cyclins and cyclin-dependent kinases (CDKs), and CDK inhibitor decreased SOX2 expression and inhibited sphere formation [199].

Some inhibitors have been found to down-regulate SOX2. PTPRZ and PTPRG, both belong to receptor-type protein tyrosine phosphatases (RPTPs), and are high expressed in sphere forming cells. Depletion of PTPRZ by knockdown or inhibitor NAZ2329 decreases SOX2 expression and sphere formation [200]. Recently, a drug screen in human TMZ-resistant GICs was performed and a new pyrimidine synthesis inhibitor 10580 was identified to antagonize the pyrimidine synthesis enzyme dihydroorotate dehydrogenase (DHODH). In addition, this compound induces cell cycle arrest, apoptosis and GCSs differentiation by enhancing nuclear export of SOX2 [201]. Since the normal tissue and brain cells use salvage pathway for pyrimidine synthesis, this compound 10580 is suggested to be a glioblastoma therapy without side effects on normal cells [201].

In summary, SOX2 plays an important role in normal embryonic development and in adult tissue homeostasis. The absence of SOX2 would result in embryonic lethality. Moreover, SOX2 is a critical molecule during tumor development, although it may have opposed functions in different tumors. In glioblastoma, SOX2 is involved in gliomagenesis, tumor progression and therapy resistance via different pathways. Many factors regulate tumor progression via regulation of *SOX2* and stabilization of SOX2. In summary, deeper understanding of the molecular and signaling pathways mediated by SOX2 may help to improve glioblastoma treatment.

## 6. SFRP2 IN NORMAL DEVELOPMENT AND CANCER

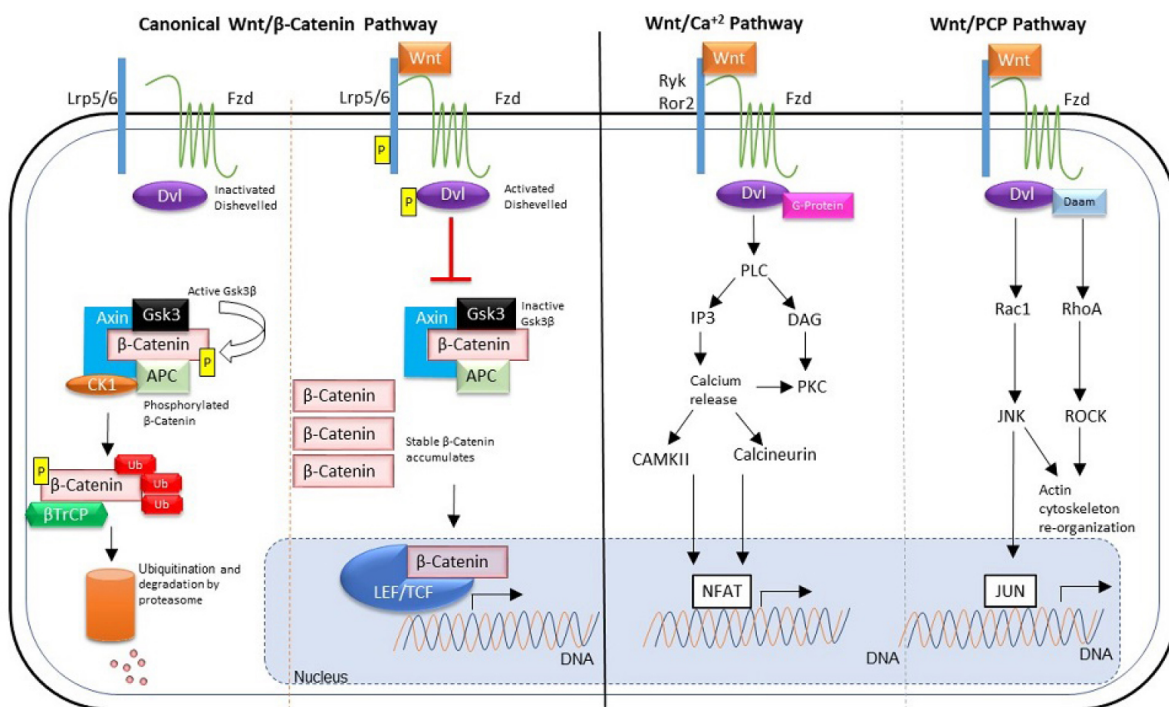
SFRP2 is a secreted frizzled-related protein and contains a putative signal sequence, a frizzled-like cysteine-rich domain (CRD), and a conserved hydrophilic carboxy-terminal Netrin-like domain (NTN). CRD domain is homologous to the sites in Frizzled receptors that bind with Wnt proteins. SFRP2 has been shown to bind Wnt ligands or Wnt-receptor complex in different contexts, and exhibits either a promoting or an inhibitory role in Wnt signaling.



*Figure 4. Schematic diagram for structure of SFRP2.*

### 6.1 CANONICAL AND NON-CANONICAL WNT SIGNALING

There are three different main Wnt pathways, including canonical Wnt/ $\beta$ -catenin cascade, noncanonical Wnt/ $\text{Ca}^{2+}$  and noncanonical Wnt/planar cell polarity (PCP) pathways [202] (Figure 5).



*Figure 5. A schematic illustration representing canonical and non-canonical WNT signaling pathways.* Adapted from [202], with permission.

In the canonical Wnt/ $\beta$ -catenin pathway, Wnt ligands bind with Frizzled (Fz) receptor proteins and LRP5/6. Fz proteins are seven-pass transmembrane receptors with an extracellular N-terminal cysteine-rich domain (CRD). LRP5/6 is a single-pass transmembrane molecule. Upon Wnt ligand binding to the Fz receptor, the receptor interacts with and

phosphorylates Disheveled (Dsh) and GSK3 $\beta$ . GSK3 $\beta$  and casein kinase 1 $\alpha$  (CK1 $\alpha$ ), subsequently induce the phosphorylation of LRP, and regulate Axin docking, resulting in the nuclear translocation and stabilization of  $\beta$ -catenin. Then  $\beta$ -catenin binds with the transcription factor complex T-cell factor (TCF)/lymphoid enhancer factor (LEF), and induces the transcriptions of genes. In the absence of Wnt, GSK3 $\beta$  and CK1 $\alpha$  phosphorylate  $\beta$ -catenin and induce ubiquitination and degradation of  $\beta$ -catenin. In the nucleus, Groucho, instead of  $\beta$ -catenin, binds with TCF/LEF transcription factor and inhibits the transcription of Wnt target genes [202].

Many proteins have been reported to inhibit the canonical Wnt signaling pathway. The secreted Dickkopf (DKK1) is a secreted protein that antagonizes the Wnt pathway by binding with LRP6 and promoting LRP6 internalization and degradation to interrupt the Wnt-Fz-LRP6 complex [203]. ROR2, another tyrosine kinase receptor with CRD motif, has been shown to bind with Wnt5a and inhibit the binding of Wnt with Fz receptors. Wnt5a/Ror2 pathway represents a non-canonical Wnt signaling pathway [204]. Soluble Frizzled-Related Proteins (SFRPs) resemble the ligand-binding CRD domain, and were at the beginning presented as Wnt inhibitors by competing with Fz receptors for Wnt binding, such as Wnt1 and Wnt8, but not Wnt5a [205, 206]. SFRPs have also been shown to enhance of the canonical Wnt/ $\beta$ -catenin pathway by direct interaction with Fz receptors or increase extracellular export of Wnt proteins [207]. SFRP2 has been reported to enhance Wnt-3a-dependent LRP6 phosphorylation and  $\beta$ -catenin translocation to the nucleus [208].

Both non-canonical Wnt pathways do not involve  $\beta$ -catenin. Wnt/Ca<sup>2+</sup> pathway controls the release of Ca<sup>2+</sup> from the endoplasmic reticulum (ER) to cytosol. In this pathway, Wnt binding leads to the activation of either phospholipase C (PLC) or cGMP-specific phosphodiesterase (PDE). Activation of PLC induces IP3 cleaved from PIP2, and IP3 induces Ca<sup>2+</sup> release after binding the receptor. Increased Ca<sup>2+</sup> activates calcineurin and CaMKII, which activate NFAT, and affects cell adhesion, migration and tissue separation. However, activation of PDE has an inhibitory function in Ca<sup>2+</sup> release by inhibition of protein kinase G (PKG) [202].

Wnt/PCP pathway controls the polarization of epithelial cells along the plane of the basal membrane. In this pathway, Wnt binds to Fz and other receptors, such as NRH1, Ryk, PTK7 or ROR2, and recruits Dsh. In one way, Dsh forms complex with Disheveled-associated activator of morphogenesis 1 (DAAM1) and activates the small G-protein Rho and Rho-associated kinase (ROCK). In another way, Dsh forms complex with RAC1 and activates JNK. Both ways result in regulation of the cytoskeleton [202].

Wnt3a, Wnt5a and Wnt7a are three most important Wnt family members. Wnt3a is a ligand that activates  $\beta$ -catenin signaling to induce expression of multiple genes for cell proliferation, such as c-Myc, CD44 and SOX9. Wnt5a mediates non-canonical Wnt/PCP signaling, and has been shown involved in atherosclerosis and vascular thrombosis [209]. Wnt7a is a most

studied Wnt ligand. It plays a role in both  $\beta$ -catenin and PCP signaling, and functions in embryogenesis and is involved in the pathogenesis of many cancers [210, 211].

## 6.2 WNT SIGNALING AND GLIOBLASTOMA

Aberrant Wnt signaling is a driving force in many cancers. Many reports have shown the correlation of Wnt signaling with GSCs. FAT atypical cadherin 1 (FAT1) is a cadherin-like tumor suppressor protein that can bind with  $\beta$ -catenin and suppress its nuclear translocation. Somatic mutations of FAT1 have been reported to occur in 20.5% of glioblastoma. Inactivation of FAT1 promoted Wnt/ $\beta$ -catenin and tumorigenesis in glioblastoma [102]. Wnt is also connected with other pathways, such as EGFR, and HGF pathways. c-Met is a receptor of HGF. It has been shown that GSCs with higher expression of c-Met have more activation of Wnt/ $\beta$ -catenin signaling [106]. Pleiomorphic adenoma gene like-2 (PLAGL-2) has been found high expressed in glioblastoma, and to activate the WNT/ $\beta$ -catenin pathway in GSCs. Overexpression of PLAGL-2 suppressed stem cell differentiation and induced the expression of stem cell marker Nestin [212]. Dickkopf (DKK1) is an inhibitor of Wnt signaling and suppression of DKK1 in GSCs promotes Wnt signaling. ASCL1, a transcription factor that is involved in GSC maintenance and propagation, has been shown to activate Wnt signaling by suppression of DKK1 [213]. Frizzles-related proteins (FRPs) are soluble proteins that can interfere the binding of Wnt with frizzles receptors (Fzd). The gene promoter hypermethylation of Wnt inhibitory proteins, such as sFRP1, sFRP2 and Naked (NKD2), has been shown to occur in 40% of primary glioblastoma [214]. Wnt inhibitory factor 1 (WIF1) could induce cell senescence and impede stemness and tumor growth [215].

Wnt signaling is also involved in tumor invasion. Overexpression of active  $\beta$ -catenin in glioblastoma cells increased ZEB1 expression and cell motility. Conversely, suppression of  $\beta$ -catenin in U87MG and LN229 suppressed tumor cell invasion [216]. In addition, knockdown of Wnt5a inhibited cell migration and recombinant Wnt5a stimulates migration by increasing the activity of MMP2 [217]. Oligodendrocyte precursors (OPCs), which express Olig2, PDGFR $\alpha$ , NG2 and other markers, are one source of glioblastoma. It has been reported OPCs produced Wnt7 signaling and affected the vascularization [218]. In addition, the interaction between OPCs and endothelial cells promoted OPCs migration via Wnt-CXCR4 signaling [219].

Wnt signaling also contributes to treatment resistance of glioblastoma. It has been reported there are more cells with both active  $\beta$ -catenin (ABC) and SOX2 (ABC<sup>+</sup>/SOX2<sup>+</sup>) in radioresistant glioblastoma cells. The amount of these cells increased with additional radiotherapy, but cells became radiosensitive when using Wnt pathway inhibitor XAV 939 or siRNA [220], suggesting the combinational treatment of radiotherapy and inhibition of Wnt signaling should be considered in further treatment.

Many drug that target Wnt pathway has been tested in glioblastoma. Aspirin, a non-steroidal anti-inflammatory drug (NSAID), can inhibit cell proliferation and invasiveness by decreasing TCF/LEF promoter activity and target genes expression (c-MYC, Cyclin D1, and FRA-1) [221]. Diclofenac and Celecoxib, another two NSAIDs that can inhibit COX-1/2, have been shown to reduce the glioblastoma growth [222]. Both SEN461 and XAV939 are potent WNT signaling inhibitors, that can stabilize AXIN, increase phosphorylation and degradation of  $\beta$ -catenin [223]. However, their clinical effects have not been reported.

### **6.3 SFRP2 AS AN ANTAGONIST OF WNT SIGNALING**

SFRP2 is primarily reported as a Wnt signaling inhibitor by preventing the binding of Wnt with Fz receptors [224]. SFRP2 enhances osteogenic differentiation potential by suppression of WNT1 and antagonizing the canonical WNT pathway [225]. In the progression of chronic obstructive pulmonary disease (COPD), exposure to cigarette smoke extract (CSE) induces *SFRP2* expression, and SFRP2 further increases airway inflammation by inhibition of  $\beta$ -catenin [226].

SFRP2 has been identified as tumor suppressor in many cancers with low expression level or hypermethylation of *SFRP2*. Down-regulation of SFRP2 shows a potent tumor-promoting effect in choriocarcinoma (CC). Decreased SFRP2 promotes cancer stemness and the epithelial-mesenchymal transition (EMT) process via activation of Wnt/ $\beta$ -catenin signaling [227]. Hypermethylation of *SFRP2* is detected in stool and suggests to be a potential marker for colorectal cancer and precancerous lesions [228]. Hypermethylation of *SFRP2* is also a frequent alteration in breast cancer [229], gastric cancer [230], and cervical cancer [231].

SFRP2 can also promote tumors as a Wnt antagonist. In melanoma, SFRP2 was found to be secreted by aged fibroblasts and promote angiogenesis, tumor metastasis and treatment resistance, where SFRP2 functions as a Wnt antagonist, and decreased  $\beta$ -catenin and APE1, which renders melanoma cells more resistant to the targeted therapy [232]. In P53 mutant-osteosarcoma (OS) patients' samples, high SFRP2 has been shown to correlate with poor survival. Ectopic SFRP2 overexpression in osteoblast precursors promoted OS phenotype via increase of oncogenic *FOXMI* and *CYR61* expression. SFRP2 overexpression cells showed a decrease in Wnt3a-mediated canonical Wnt [233].

### **6.4 SFRP2 AS AN AGONIST OF WNT SIGNALING**

In many studies, SFRP2 is described to act as an agonist of Wnt signaling. SFRP2 regulates the extension of anaerobic glycolysis in mouse cardiac fibroblasts (CFs) through canonical Wnt/ $\beta$ -catenin signaling by increasing Wnt3a [234]. SFRP2 can also activate the non-canonical Wnt pathway during vertebrate gastrulation, where SFRP2 promotes Ror2 signaling by stabilizing a Wnt5a-Ror2 complex [235]. In the damaged tumor microenvironment, DNA damage of stromal cells activates NF- $\kappa$ B, which binds to the *SFRP2*

promoter and increases SFRP2 expression. Then SFRP2 can enhance Wnt16B/ $\beta$ -catenin signaling and promote therapeutic resistance [236].

SFRP2 has also been reported as a WNT agonist and promotes growth in some tumors. In renal cancer, SFRP2 has an oncogenic function and activates the canonical WNT pathway by decreasing the phosphorylation of  $\beta$ -catenin [237]. In breast cancer, SFRP2 was found to induce endothelial tube formation and angiogenesis via activation of the calcineurin/NFATc3, a non-canonical WNT pathway [238]. SFRP2 is also involved in the lung metastasis of breast cancer. The crosstalk between the cancer cells and lung epithelial cells induces SFRP2 expression in breast cancer cells, and promotes fibrillogenesis, which gives a support to the regrowth of cancer cells [239]. Moreover, SFRP2 could be considered as a potential therapeutic target. Fontenot et al. have shown that treatment with SFRP2 monoclonal antibody decreased tumor volume in mice allografts with breast cancer cell MDA-MB-231, where they found the decreased activation of  $\beta$ -catenin and NFATc3, indicating SFRP2-Wnt signaling is important in breast cancer [240].

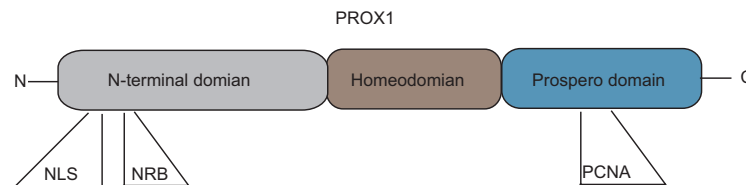
## 6.5 SFRP2 AND GLIOBLASTOMA

The involvement of SFRP2 in glioblastoma has not been extensively explored. Majchrzak-Celińska et al. tested the promoter methylation of WNT pathway regulators, including *SFRP1*, *SFRP2*, *DKK1*, *PPP2R2B*, *SOX17* and *DACH1* in 64 glioma samples, which contained 26 glioblastomas. They found that methylation of *SFRP1* and *SFRP2* was frequently occurring, with respectively 73.4% and 46.9%. But only *SFRP1* promoter methylation predicted worse patient survival [241]. In one report ectopic expression of SFRP2 in glioma cells enhanced clonogenicity and resistance to serum starvation, as well as inhibited cell mobility by decreasing the expression of matrix metalloproteinase-2 (MMP-2) and tyrosine phosphorylation of  $\beta$ -catenin [214]. Conversely, SFRP2 has been shown as a tumor suppressor in another report, where overexpression of SFRP2 inhibited glioblastoma sphere formation and tumor growth *in vivo*. Mir22HG, which can target SFRP2, was found highly expressed in glioblastoma. Suppression of Mir22HG attenuated cell proliferation and tumor growth by inhibiting Wnt/ $\beta$ -catenin signaling, indicating SFRP2 is a Wnt antagonist in this context [242].

Above all, the effect of SFRP2 in different cells is distinct, and context depended, underlying these reports stating different activities. It is still inconclusive if SFRP2 acts as a WNT agonist or antagonist in glioblastoma. The net *in vivo* effect of SFRP2 in glioblastoma is thus still a very open question. But in support of further studies, it does affect key glioblastoma phenotypes, and is differentially expressed between glioblastoma cases.

## 7. PROX1 IN NORMAL DEVELOPMENT AND CANCER

The Prospero-Related Homeobox 1 (PROX1) is a transcription factor, a protein of 82.3 kDa. The N-terminal part of PROX1 harbors a nuclear localization signal (NLS) and two nuclear receptor boxes (NRB). The prospero-domain in C-terminal contains a functional proliferating cell nuclear antigen (PCNA) interacting motif. PROX1 is essential for the development of several organs and knockout of *Prox1* in mice embryos results in lethality due to multiple developmental defects [243].



**Figure 6.** Schematic diagram for structure of *PROX1*.

### 7.1 PROX1 IN NORMAL DEVELOPMENT

PROX1 has been shown to be critical in the development of several organs, especially in lymphatic system [243]. Many publications have reported the regulation of PROX1 during lymphatic development. PROX1 is a lymphatic endothelial cell (LEC) master transcription factor, where Wnt/ $\beta$ -catenin signaling is important during lymphatic vessels development. PROX1 has been shown to form a complex with  $\beta$ -catenin and enhance its signaling [244]. Together VEGFC, FLT4 and PROX1 could be involved in angiogenic sprouting and lymphatic formation, with haematopoietically expressed homeobox (HHEX) as their upstream regulator [245]. Moreover, depletion or hyper-activation of LEC-specific Hippo pathway molecular YAP/TAZ aggravates or attenuates the lymphatic formation by increasing or decreasing PROX1 transcription [246]. In addition, PROX1 is important in other organs. PROX1 can regulate sarcomere integrity and maintain the function of the heart [247]. It is also essential for skeletal muscle phenotype and myoblast differentiation by cooperation with the NFAT and NOTCH1 pathways [248].

PROX1 can be detected in different stages of brain development. At the prenatal stage in mouse, *Prox1* is detected in subventricular zone and other regions in prethalamus and hypothalamus. *Prox1* can be found in thalamus, the cerebellum, and the hippocampus in postnatal stage. However, *Prox1* only remains its expression in the hippocampus and cerebellum in the adult. These different distributions of *Prox1* indicates its role in brain development [249]. PROX1 was shown to be important in CNS development and neurogenesis by induction of differentiation of neural stem cells [250].

Many factors regulate or are regulated by PROX1 during neurogenesis. SoxC proteins, such as Sox4 and Sox11, were reported to bind with the promoter of PROX1 and activate PROX1 in neurogenesis [251]. NOTCH1 signaling is important in the self-renewal of neural

progenitor cells (NPCs). PROX1 was shown to be induced in differentiated NPCs and inhibit NOTCH1 signaling, that is to maintain the balance between cell self-renewal and differentiation [252]. PROX1 is a target of  $\beta$ -catenin signaling. PROX1 enhances neuronal differentiation but is not required for survival of mature granule cells [253]. PROX1 has also been shown to be important for induction of neurogenesis in the dentate gyrus (DG), and has been shown to be indispensable in oligodendroglialogenesis in the SVZ [254].

Moreover, PROX1 has been shown to regulate the circadian rhythm and energy metabolism. It has been shown that PROX1 can form a complex with the estrogen-related receptor  $\alpha$  (ERR $\alpha$ ), BMAL1 and PGC1 $\alpha$  and repress their transcriptional activity [255]. PROX1 also repress the activity of the retinoic acid-related orphan receptors (ROR $\alpha$  and ROR $\gamma$ ). Knockdown of PROX1 increases ROR $\gamma$  and its target clock genes *Bmal1*, and cryptochrome 1 (*Cry1*), to control the clock and metabolic network [256]. Related to energy metabolism, it has been reported that the rs340874 single nucleotide polymorphism (SNP) in *PROX1* is significantly correlated with the insulin level in the plasma, and the knockdown of *PROX1* decreased the insulin secretion, suggesting reduced PROX1 expression is susceptible for type II diabetes [257].

## 7.2 PROX1 AND CANCER

PROX1 is involved in tumorigenesis and progression, but has been reported to exert both oncogenic and suppressive properties. It promotes tumor progression and invasion in colon cancer cells [258, 259]. In the colorectal cancer (CRC), the loss of *APC* or *CTNNB1* has been reported as the initial mutations, leading to activation of canonical Wnt/ $\beta$ -catenin pathway. Then elevated Wnt activity induces PROX1 expression. PROX1<sup>+</sup> cells were found with stem cell property in intestine adenomas, but not in normal intestine. PROX1 is important for the maintenance of stem cell population, and it inhibits annexin A1 to increase the expression of filamin A, which plays a role in DNA damage repair [259]. In addition, PROX1 can modulate metabolic adaption, and promotes colon cancer cell proliferation during hypoxia and nutrient deprivation, by suppressing the pro-apoptotic molecule BCL2L15 [258]. PROX1 also interacts with nucleosome remodeling and deacetylase (NuRD) complex to suppress NOTCH1 pathway and differentiation in colon CSCs [260]. In breast cancer, PROX1 has been shown with tumor suppressive function that PROX1 is hyper-methylated and transcriptionally silenced [261]. Moreover, PROX1 inhibits tumor in esophageal cancer [262], pancreatic cancer [263], and neuroblastoma [264]. In hepatocellular carcinoma (HCC), the function of PROX1 is controversial. PROX1 has been shown with a correlation with differentiation score in the tumor, and the low expression was associated with bad prognosis. Suppression of PROX1 accelerated tumor cell growth [265]. It has reported that PROX1 inhibits Twist expression, which binds with E-box of P53 promoter. Therefore PROX1 was suggested to inhibit tumor growth by induction of P53-dependent senescence [266]. Mir-670-5p, which targets on PROX1, has been shown high expressed in HCC, and suppressed PROX1 expression to promote cell proliferation [267]. Conversely, in another report PROX1



expression in primary HCC has been shown correlated with worse survival. Knockdown of PROX1 suppressed cell invasion and tumor metastasis in mice xenografts, where PROX1 up-regulated HIF expression and stabilized HIF-1 $\alpha$  to promote epithelial–mesenchymal transition [268]. Moreover, PROX1 can enhance  $\beta$ -catenin activation and promote sorafenib resistance in HCC cells [269]. It is difficult to explain the opposite results. One reason may be different HBV infection states in HCC patients.

Until now, there are not many reports on the function of PROX1 in glioblastoma. PROX1 has been detected in gliomas of different grades. By using immunohistochemistry, Elsir et al. showed 79% of Grade IV, 57% of Grade III, and 21% of Grade II gliomas contain cells that are strongly PROX1 positive. PROX1 was co-expressed with neural protein marker MAP2 and  $\beta$ III-tubulin in these cells, and these cells also had lower proliferation rates [270]. In grade II glioma, higher PROX1 is a predictor for worse survival, combined with IDH mutation and loss of chromosomal arms 1p/19q [271]. In grade IV, PROX1 was shown to have no correlation with survival in the primary glioblastomas, but higher PROX1 is a prognostic marker in the secondary glioblastomas with IDH-mutation and 1p19q non-codeletion [272]. Another report showed overexpression of PROX1 in glioblastoma cell line increased cell proliferation and xenograft growth by activating NF- $\kappa$ B pathways [273]. These findings highlight the role of PROX1 during gliomagenesis, and the mechanism and function of PROX1 in glioblastoma should be further investigated.

## 8. PRESENT INVESTIGATION

### 8.1 AIMS

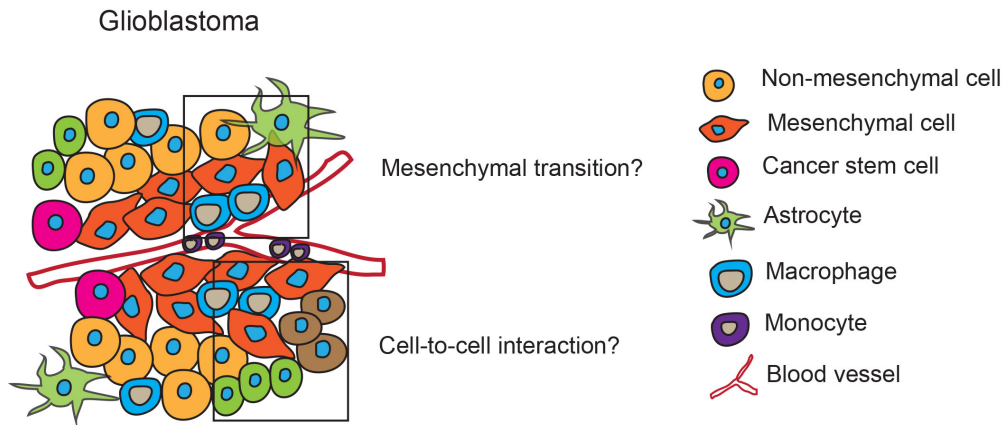
The general aim of this thesis was to investigate the mechanisms of intratumoral heterogeneity in glioblastomas: how the different glioblastoma subtypes transition, how the different clones inside the tumor communicate with each other and how the heterogeneity affects tumor treatment.

Specific aims were:

To study the regulators of glioblastoma mesenchymal transition

To investigate the function of PROX1 in glioblastoma

To identify the functionally distinct and interacting cancer cell populations in glioblastoma



*Figure 7. Schematic diagram illustrating the aims of this thesis.*

## 8.2 RESULTS

### 8.2.1 Paper I

#### **SFRP2 induces a mesenchymal subtype transition by suppression of SOX2 in glioblastoma**

Glioblastoma is an intratumorally heterogeneous tumor, with different proneural, classical and mesenchymal gene expression subtypes presented in individual tumors. A transition from non-mesenchymal to mesenchymal subtype cells has been reported to occur during tumor progression. In this study, we aimed to identify regulators that can mediate a transition between non-mesenchymal and mesenchymal gene expression subtype. We started with a CMap analysis on gene expression data from two separate datasets, 48 samples from HGCC and 45 glioblastoma cell cultures from CCLE, that were derived from cell cultures grown in defined neurosphere medium on laminin or in serum, respectively. We used this dual analysis approach to reduce the contribution of expression patterns that are connected to different culture conditions. In the CMap analyses, we identified SOX2 as a major gene expression pattern modulator for both the HGCC and the CCLE cultures in the group with non-mesenchymal gene expression signature.

We have previously defined two subsets of glioblastoma cell cultures, denoted Hesselager type A (non-mesenchymal, SOX2<sup>high</sup>) and type B (mesenchymal, SOX2<sup>low</sup>). Subsequently, we have named genes on average higher expression in type B cells as type B genes and in type A cells type A genes, respectively. In order to investigate if any of these genes specifically regulates the mesenchymal subtype, we performed an overexpression screen by overexpressing type B genes in the type A cell line U-2987, which originally has high SOX2 expression, and monitored the levels of SOX2 expression. Here SFRP2 was identified as a SOX2 suppressor. Next, we generated RNA-seq data from these cells with and without SFRP2 overexpression and analyzed. We found that SFRP2 overexpression switched the cells from a non-mesenchymal to a mesenchymal gene expression pattern. SFRP2 was also found on average higher expressed in glioblastoma mesenchymal subtype in the TCGA samples, than in the proneural and classical subtype samples. Our phenotypic assays *in vitro* with the SFRP2 overexpressing cells further showed increased cell Matrigel invasion and decreased tumor sphere formation, cell proliferation, and altered levels of cell cycle proteins. Conversely, to investigate if these phenotypic changes could be reversely recapitulated in connection to SOX2, we overexpressed SOX2 in the cell culture U-2982, which original is of mesenchymal type and thus have low SOX2 levels. We found that SOX2 overexpression induced a transition from a mesenchymal glioblastoma subtype gene expression pattern to a proneural subtype pattern, and in connection increased the tumor formation and decreased Matrigel invasion capacity. In an analysis where we divided TCGA glioblastoma samples into four groups according to the median expression values of *SFRP2* and *SOX2*, the subgroup with high *SFRP2* and low *SOX2* expression was enriched with mesenchymal

glioblastoma subtype cases and with shorter survival.

To investigate the mechanism of *SOX2* inhibition upon *SFRP2* overexpression, we integrated CCLE gene expression profiling with RNA-seq data analyses from *SFRP2* or *SOX2* overexpression cells followed by gene overexpression or knockdown modification of the potential mediator genes. *KLF4* was identified as a candidate protein involved in the suppression of *SOX2* by *SFRP2*. Using global protein tyrosine kinase analysis by Pamgene, *PDGFR* signaling was identified and we found *PDGFRA* was decreased and *PDGFRB* was increased by *SFRP2* overexpression. Since *SFRP2* has been regarded as both an agonist and an antagonist of Wnt/ $\beta$ -catenin signaling, we tested *SOX2* expression after knockdown of *CTNNB1* ( $\beta$ -catenin), however, it was apparently not involved in the inhibition of *SOX2* by *SFRP2*.

At the end, we conducted a spatial tumor tissue analysis where genes that were both positively correlated to and induced by *SFRP2* were found to be higher expressed in vascularized tumor areas, whereas *SOX2* positively correlated and induced genes were located in cellular tumor areas. Human protein atlas (HPA) staining also showed type A genes and type B genes distributed in cellular and vascular areas, respectively. In line with this, increased pro-angiogenic, pericyte and macrophage markers were identified in the vascular areas with high *SFRP2* and low *SOX2* expression. In support of the potential immunogenic effect, conditioned media from *SFRP2* overexpression cells had a relatively higher capacity to increase CD206 expression in human monocytes, which is also known to be part of an M2-polarization phenotype.

In summary, *SFRP2* was identified to induce a mesenchymal gene expression pattern by suppression of *SOX2* expression. *SFRP2*-induced and positively *SFRP2*-correlating genes were found at higher levels in the vascularized areas of tumor tissue, and *in vitro* was connected to a CD206 expression increase in macrophages. Overall, this study proposes that *SFRP2* induces a glioblastoma subtype transition that is spatially confined to vascular tumor area and provides new insight into the regulation of related gene expression patterns in intratumorally heterogeneous glioblastomas.

## 8.2.2 Paper II

### **Reduced expression of PROX1 transitions glioblastoma cells into a mesenchymal gene expression subtype**

Prospero-related homeobox 1 (*PROX1*) is a transcription factor that is expressed and functionally active in some tumors, such as in neuroblastoma where it has a growth suppressive role [264]. In this study, we aimed to investigate the functional role of *PROX1* in glioblastoma by performing integrated analyses of RNA-seq data derived from *in vitro* experiments and publicly available expression data from single cell sequencing experiments.

We started with analysis of a combined TCGA glioma data set, which contains grade II to grade IV gliomas. Grade IV gliomas (glioblastomas) showed lower expression of *PROX1* and connection with worse overall survival compared with grade II and III gliomas, which showed higher *PROX1* expression. In line with this, the group of glioblastoma patients with high *PROX1* levels also showed better prognosis and were enriched for mesenchymal glioblastoma gene expression subtype tumors. We then analyzed the PROX1 protein expression in a panel of glioblastoma cell lines, and noticed that in most cells SOX2 and GFAP were higher expressed in PROX1 expressing cells and FN1 was higher in PROX1 negative cells, with U-343 MGa and U-343 MG glioblastoma cells as examples, respectively. These two cell lines were used for subsequent generation of stable overexpression cell lines and RNA-seq analyses.

We thus overexpressed PROX1 in U-343 MG and suppressed it in U-343 MGa. RNA-seq gene expression and gene set enrichment analysis showed PROX1 was connected with neurogenesis, cell proliferation, and epithelial-mesenchymal transition signatures. By comparing PROX1 regulated genes with glioblastoma subtype classifier genes, PROX1 overexpression was found to decrease the support index for mesenchymal subtype and increase it for non-mesenchymal subtypes in U-343 MG. Conversely, PROX1 depletion in U-343 MGa increased support index for mesenchymal subtype. Moreover, we found that PROX1 was higher expressed in HGCC cultures with a higher tumorigenicity than in those with low tumorigenicity. Overexpression of PROX1 in U-343 MG and suppression of PROX1 in U-343 MGa, increased and decreased cell proliferation and the cell cycle regulators cyclin A and E, respectively. In connection to this, SOX2 was shown as a positive regulator of PROX1. SOX2 and PROX1 were positively correlated in several gene expression datasets, including CCLE, HGCC and TCGA. SOX2 increased and decreased PROX1 expression upon overexpression and depletion modification of SOX2, respectively. CVT-313, a CDK1/2 inhibitor was found to decrease both SOX2 and PROX1 expression in the glioblastoma cells.

Furthermore, we investigated the *PROX1*-related gene expression patterns, by a *PROX1* proxy signature, at single-cell level by using the single cell RNA-seq data from five glioblastomas. The *PROX1* proxy signature was developed, since *PROX1* measurement was not available in the single-cell dataset. We observed cell heterogeneity in each tumor regarding *PROX1* proxy and its correlated genes. Among the top 100 PROX1 proxy correlated gene, 71% of them could be increased by PROX1 overexpression in U-343 MG.

Finally, we investigated the interactome of PROX1. The transcription factor associated protein THRAP3 (also known as TR150) was detected in a PROX1 nuclear complex co-immunoprecipitation mass-spectrometry analysis. THRAP3 was also lower expressed in grade IV gliomas than in grade II and III. Both THRAP3 and PROX1 localized to the nucleus of glioblastoma cells, and showed a positive correlation in TCGA samples. Furthermore,

THRAP3 was identified as a negative regulator of PROX1 by suppression of THRAP3 in U-343 MGa. Since THRAP3 in other studies has been reported to bind and regulate SOX9, in this study we also found a positive correlation between *SOX2*, *SOX9*, *PROX1*, and *THRAP3* in TCGA glioblastoma cases.

In conclusion, PROX1 could be a regulator of glioblastoma subtype gene expression signature. The reduction of PROX1 induced a mesenchymal transition *in vitro* and was correlated to shorter survival in TCGA glioblastoma cases. *PROX1* expression showed heterogeneity in single cells within the tumor. Moreover, PROX1 levels could be increased upon overexpression of SOX2. PROX1 could interact with THRAP3, which has a suppressive effect on PROX1 levels. These findings provide novel insights into the mechanisms of intratumoral heterogeneity and transcription factor networks in glioblastoma.

### 8.2.3 Paper III

#### **Identification of functionally distinct and interacting cancer cell subpopulations from a glioblastoma with intratumoral genetic heterogeneity**

Glioblastoma is a heterogeneous tumor with different cell populations within individual tumors. More knowledge on the interactions between these populations will be helpful for developing new therapeutic strategies and to provide further understanding of the fundamental tumor biology. In this study, we used the U-343 cell culture model to investigate more in depth differences between subpopulations of neoplastic cell populations within a single glioblastoma and model their communication with each other. The U-343 system consists of multiple established cell lines and the cell lines used here are denoted as U-343MG, U-343MGa, U-343MGa 31L, and U-343MGa C12:6, which all originate from a single glioblastoma tumor. U-343MG and U-343MGa were originally derived from different parts of the tumor, and U-343 MGa 31L and U-343MGa C12:6 are subsequent derivatives from U-343 MGa.

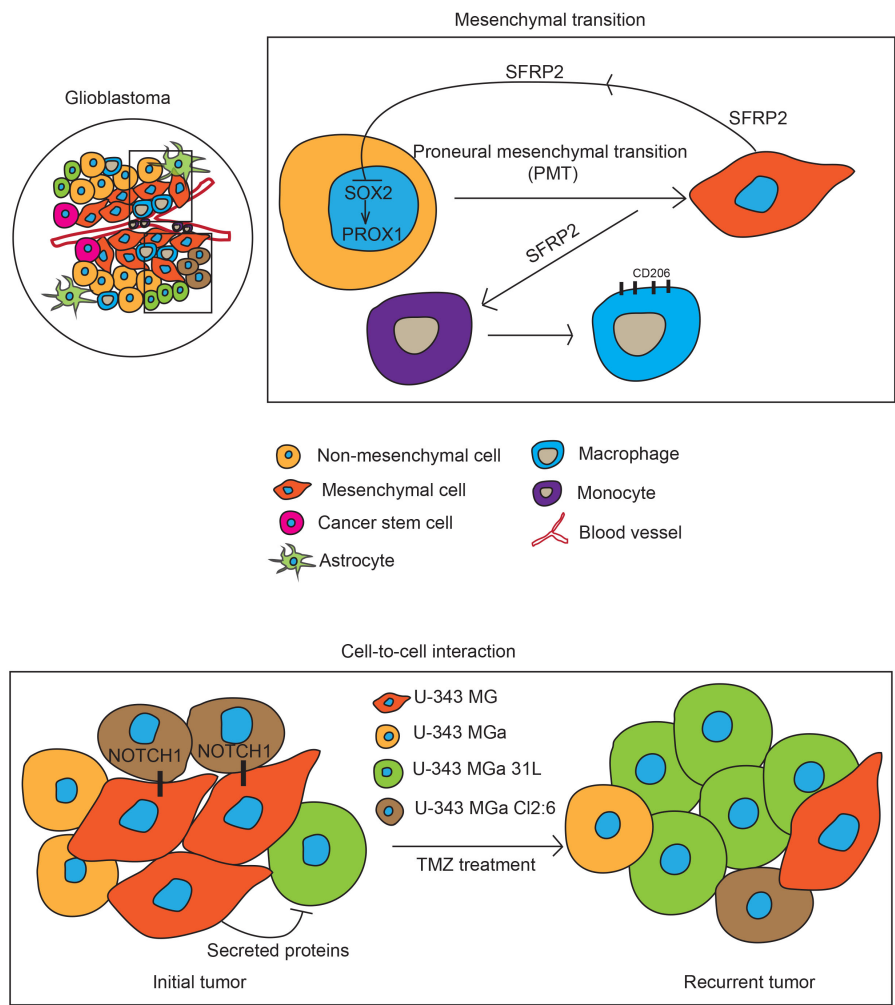
To establish the identity of the cells we first investigated the GFAP and FN1 expression patterns by immunofluorescence staining. U-343MG cells were GFAP<sup>+</sup>FN1<sup>+</sup>, whereas U-343 MGa and its subclones were GFAP<sup>+</sup>FN1<sup>-</sup> cells. STR analysis validated with a high certainty level that the cell lines were derived from a single individual. Moreover, by using copy number analysis we identified common and specific genetic alterations in the four U-343 cell lines. These cell lines showed different proliferation rates and TMZ sensitivity. To further investigate the effect of intratumoral heterogeneity on drug sensitivity, we performed co-culture experiments, mixing all four populations in equal amounts, and treated with or without TMZ for 5 and 10 days. U-343 MGa 31L was found as the least represented cell line when co-cultured with others, but during treatment with TMZ it became the most frequently occurring cell type in the co-culture.

To better understand if the U-343 model is representative of glioblastomas, we analyzed gene expression profiles from CCLE cell lines and single-cell sequencing data from individual glioblastomas, where U-343 MG and U-343 MGa cultures were shown to respectively resemble *GFAP<sup>+</sup>FNI<sup>+</sup>* or *GFAP<sup>+</sup>FNI<sup>-</sup>* cell lines and individual cells within single glioblastomas as determined by single cell RNA-seq data analysis. Further analyses by generation and analysis of RNA-seq data in combination with invasion assays illustrated that U-343 MG had a more mesenchymal phenotype than others.

Subsequently, we performed cell-to-cell contact and conditioned media experiments to study the interactions between clones. In cell-to-cell contact experiments, one GFP-labeled cell line was seeded relatively sparse on the top of another cell line. We especially found that U-343 MGa Cl2:6 proliferated faster when growing on top of U-343 MG. Morphological observations and qPCR results from sorted GFP-labeled cells suggested a transition to a more mesenchymal gene expression based on increased expression of *FNI* and *SNAI2*, and decreased levels of *SOX2* and *GFAP*. To investigate the growth promoting mechanism affected by the cell-to-cell interaction, the involvement of NOTCH1 was tested, based on the notion of differential expression levels of NOTCH receptors and ligands obtained from RNA-seq data and Westernblots. However, upon CRISPR-Cas9 knockout or shRNA- knockdown of NOTCH1 in U-343 MGa Cl2:6, its proliferation was further enhanced during co-culture with U-343 MG. As a second way of investigating interclonal communication, we focused on interactions between cells mediated by secreted proteins. Conditioned media from U-343 MG was found to have a growth inhibitory effect on U-343 MGa 31L. By generation and a combined analysis of mass spectrometry data of the cells secretome and RNA-seq data from the U-343 cells, TGFBI and ADAMTS1 were identified as highly expressed in U-343 MG, and therefore selected for further experiments by knock-down or use of recombinant protein to investigate their involvement in the co-culture proliferation phenotype of U-343 MG and U-343 MG 31L.

In summary, this study illustrates that the cell populations within the individual heterogeneous tumor have common and specific properties. They communicate with each other by cell-to-cell contacts and by secreted proteins and this heterogeneity affect TMZ treatment results and recapitulate a tumor relapse phenotype. Thus, the U-343 model provides evidence for the complexity and importance of intratumoral heterogeneity in glioblastoma, and constitutes a suitable model system for inter-cellular signaling studies inside glioblastoma.

8.2.4 Concluding graph



**Figure 8. Schematic diagram for the conclusions of this thesis.** Upper figure shows that SFRP2 is secreted by mesenchymal subtype glioblastoma cells, and induces proneural to mesenchymal transition (PMT) of the non-mesenchymal cells by suppression of SOX2 and PROX1. In addition, the conditioned media from SFRP2-overexpressing cells increases CD206 expression in macrophages. Lower figure shows the cell-to-cell interactions in the U-343 cell system by direct and indirect cells communication. U-343 MG increases the proliferation of U-343 MGa C12:6 via cell-to-cell contact, and it can also inhibit the proliferation of U-343 MGa 31L via secreted proteins.



## 8.3 DISCUSSION AND FUTURE PERSPECTIVES

Glioblastoma is an aggressive disease with poor prognosis. Intratumoral heterogeneity is a characteristic hallmark that is thought to play an important role in glioblastoma progression. Better understanding of how heterogeneity appears and of the related consequences is thought to provide crucial biological insight to this devastating disease and provide ideas on novel treatment targets. In this thesis work, we set out to identify regulators of glioblastoma subtype gene expression states during tumor evolution (Paper I and II) and to investigate interclonal interactions in glioblastomas and relapse due to clonal variation in drug sensitivity (Paper III).

### 8.3.1 SOX2 is a critical factor for the maintenance of a non-mesenchymal glioblastoma phenotype

SOX2 is essential for embryonic stem cells and can be found expressed in neurogenic regions in human brain subventricular zone. In cancer, SOX2 has been reported as a key maintainer of the stem cell phenotype. The function of SOX2 in maintenance of non-mesenchymal glioblastoma phenotypes was also investigated with CMap analyses as shown in paper I. We performed hierarchical clustering using CCLE and HGCC publicly available dataset and identified major gene expression based subgroups. Subsequently transcription factors predicted to contribute to these differences in expression were analyzed by CMap, where SOX2 emerged as a key regulator. We also found *SOX2* was higher expressed in glioblastoma tissue from non-mesenchymal subtype cases than in tissues from mesenchymal cases, which supported the experimental findings and the subtype regulatory role of SOX2 in glioblastoma.

Herein, SFRP2 and PROX1 were identified as factors acting upstream and downstream of SOX2 respectively. SFRP2 is a secreted protein that has been described to act as an inhibitor of Wnt signaling and resulting in increased  $\beta$ -catenin phosphorylation and degradation. No publication has shown the connection between SFRP2 and SOX2. We initially found SFRP2, among several other genes, to be highly expressed in mesenchymal glioblastoma cell cultures, and subsequently it was found to have the capacity to decrease *SOX2* expression, by using an overexpression screen. Furthermore, we found that a subset of TCGA glioblastoma cases with *SFRP2*<sup>high</sup> and *SOX2*<sup>low</sup> expression was enriched with mesenchymal subtype cases, supporting our finding that SFRP2 can induce a mesenchymal transition by suppression of SOX2 (Paper I). PROX1 is a transcription factor that was shown less expression on mRNA level in glioblastomas as compared to grade II and III gliomas. In paper II it was found as a downstream factor of SOX2 based on gene expression profiling. Published CHIP-seq data has described the PROX1 gene has a SOX2 binding site, thus supporting a direct transcriptional regulation. PROX1 was positively correlated and increased by SOX2. In addition, PROX1 was shown to bind with THRAP3, which also regulated GFAP level. According to the subtype information in TCGA database, both high *SFRP2* samples and low *PROX1* samples were found in glioblastoma mesenchymal subtype, suggesting the increase

of SFRP2 or decrease of PROX1 was related with mesenchymal transition. By RNA-seq analysis both overexpression of SFRP2 and suppression of PROX1, were shown to increase the support index for a mesenchymal gene signature. In a contrary manner, overexpression of SOX2 or PROX1 decreased the support index for a mesenchymal subtype signature (Paper I and II).

The critical role of SOX2 as a regulator of glioblastoma subtype was confirmed in the cell-to-cell contact model using the U-343 system (Paper III). U-343 cell line model system is composed of four different cell lines that originated from the same tumor. U-343 MGa C12:6 is an astrocytic-like cell line, and it was switched to mesenchymal-like when growing on top of the mesenchymal cell line U-343 MG, in conjunction with the suppression of SOX2 expression. These findings emphasize that loss of SOX2 is important for the mesenchymal transition.

Mesenchymal transition has been reported in many situations in glioblastoma. Depletion of *NF1* in proneural cells induces a mesenchymal subtype transition [79]. Recurrent glioblastomas are often of mesenchymal gene expression phenotype after treatment such as anti-angiogenic therapy [84]. Stimuli from the microenvironment, such as via hypoxia or residing immune cells, can also induce a mesenchymal subtype transition. There are several master regulators of the mesenchymal gene expression signature including *STAT3*, *CEBPB*, *FOSL2*, *ZBTB18 (ZNF238)*, *RUNX1*, and *TAZ* [86]. In our studies we show that suppression of SOX2 or PROX1 induces a mesenchymal glioblastoma subtype transition. With respect to these factors, RNA-seq analysis reveals that overexpression of SOX2 decreased *CEBPB*, and overexpression of PROX1 decreased *RUNX1*, indicating that SOX2 and PROX1 may affect the expression of different mesenchymal regulators that are important during the transition, and may lay at different levels in the transcriptional regulation tree. PROX1 is proposed by us to be active downstream of SOX2, and both SOX2 and PROX1 are transcription factors that are essential for tissue development. Additional RNA-seq experiments should be performed to identify common and specific cell signaling pattern altered by SOX2 and PROX1 during the mesenchymal transition.

Many factors have been shown involved in regulating stemness via SOX2 including pathways involving WNT/ $\beta$ -catenin, NOTCH, and Hippo. In paper I, we found that three type B genes, *SFRP2*, *KLF4* and *RGS5*, were able to down-regulate SOX2 expression in an overexpression screen. Since SFRP2 is a secreted protein and thus may have the capacity to affect SOX2 levels in surrounding cells in a setting of intratumoral heterogeneity, we selected SFRP2 for further investigations. SFRP2 acted as a Wnt/ $\beta$ -catenin antagonist in our cells system since  $\beta$ -catenin levels decreased upon SFRP2 overexpression. However, the changes of  $\beta$ -catenin could not be directly connected with the decreased SOX2 levels, since suppression of  $\beta$ -catenin did not *per se* affect SOX2 levels. Thus the involvement of non-canonical WNT-signaling pathway, including ROR2 and NFAT, will be investigated in continued studies. Furthermore, SFRP2 increased and decreased the expression of PDGFRB

and PDGFRA, respectively, which led us to investigate signaling downstream of the PDGFRs, including the AKT signaling pathway. AKT phosphorylation has previously been reported to regulate SOX2 in esophageal cancer [274]. In preliminary data (that will be included in later versions of paper I), overexpression of SFRP2 indeed decreased phosphorylation of AKT, and treatment with the AKT inhibitor MG2206 on U-2987 and U-2997 suppressed SOX2 expression. This proposes a mechanism where SFRP2-AKT-SOX2 constitutes a signaling axis in the case of mesenchymal transition induced by SFRP2. Further investigations are conducted with a focus on the mechanisms by which SFRP2 may regulate AKT activity.

KLF4 is one of the four Yamanaka factors that together with SOX2, OCT4 and c-MYC are able to reprogram cells to iPS cells [73]. Notably in paper I, KLF4 that was part of the previously identified type B genes, was here found in the overexpression screen to be able to suppress SOX2. Furthermore, SFRP2 was found to have the capacity to increase the expression of KLF4. This suggests that KLF4 may act as an intermediate factor between SFRP2 and SOX2. According to the data from publicly available TCGA glioblastoma cases, KLF4 is positively and negatively correlated with SFRP2 and SOX2, respectively. These findings pose the question of how the Yamanaka factors cooperate during cell reprogramming, and in the situation of normal development: if KLF4 actually has an inhibitory effect on SOX2 during normal development. The Yamanaka factors are most often studied during overexpression, which overrides normal gene regulatory effects. Besides, how KLF4 suppresses SOX2 is another question we need to answer: does KLF4 bind directly to the SOX2 promoter? In our preliminary data, we found that KLF4 overexpression decreases AKT phosphorylation, as similar to SFRP2. The possibility that KLF4 binds directly to the SOX2 gene and suppresses its transcription is being investigated by ChIP.

RGS5, regulator of G protein signaling 5, belongs to the GTPase activator family. RGS5 has been studied in vascular biology where it influences the angiogenesis during tumor progression, and furthermore has been used as a marker for pericytes. In paper I, RGS5 was found together with SFRP2 and KLF4 to be able to downregulate SOX2 expression. In the TCGA database, we found RGS5 to be negatively correlated with SOX2 in the classical subtype but not in the proneural and mesenchymal glioblastoma subtypes. Additional studies on RGS5 in glioblastoma would be of interest.

Mesenchymal transition was found in this thesis work to be induced either by proteins secreted from mesenchymal cells including SFRP2 in paper I, or by cell-to-cell interactions with mesenchymal cells, such as during the direct cells contact between U-343 MGα C12:6 and U-343 MG in paper III. These, and other findings suggest that initially non-mesenchymal cells transition to a mesenchymal state induced by certain signals. What the underlying trigger of these signals is remain to be better understood. It may be genetic in the case of loss of *NF1*. But since NF1 is not lost in all mesenchymal subtype cells, it must also be due to other stimuli, potentially microenvironmental cues triggered by hypoxia, inflammation tumor

state, wound healing effects, or certain secreted factors or cell-to-cell contacts where non-mesenchymal glioblastoma cells are induced to transition to a mesenchymal subtype by other cells. These alternative reasons are consistent with the previous reports that mesenchymal subtype occurs at a later stage during tumor evolution.

### 8.3.2 The effect of heterogeneity on glioblastoma cell proliferation and cell cycle

Another important finding in this thesis work is the connection between glioblastoma subtype transitions and the change of proliferation rate and levels of cell cycle related proteins. In paper I and II, overexpression of SFRP2 or suppression of PROX1 decreased cell proliferation and expression of cyclins, such as Cyclin E. Recently it was reported that phosphorylation of SOX2 by a Cyclin E- and CDK2-complex lead to stabilization of SOX2. Thereby, treatment with CVT-313, an inhibitor of CDK1/2 kinase activity, leads to destabilization and degradation of SOX2 [199]. In our work (Paper II), CVT-313 treatment, or SOX2 suppression, both also led to decreased PROX1 levels. Thus, this connects glioblastoma subtype regulation and cell cycle. Though it still remains to study if direct modulation of cell cycle components, including cyclin E, can induce a subtype transition. In connection to this, others have reported that cell cycle was extended during differentiation of neural precursors and neurogenesis by increased expression of anti-proliferative genes, such as *CDKN2A* (p16<sup>INK4A</sup>), *CDKN2D* (p19<sup>ARF</sup>), and *CDKN1A* (p21<sup>Cip1</sup>). Bmi1, a member of polycomb group (PcG) proteins which mediates suppression of *Ink4a/ARF* cell cycle inhibitory proteins, p16 and p19, has been shown essential for self-renewal of neural precursors [275]. This suggests that the mesenchymal subtype cells represent in fact more differentiated and proliferate less as compared to non-mesenchymal subtype cells.

The changes in cell proliferation point to a connection with metabolism. It has been shown that in cellular reprogramming to iPSCs requires a shift from oxidative phosphorylation (OXPHOS) to glycolysis to produce more lactate, which is insensitive to O<sub>2</sub> levels [276]. In connection to this, nothing is really known about metabolic differences between glioblastoma subtype cells. In preliminary studies (data not included in thesis) we have found that SOX2 overexpression changes the mitochondrial morphology and decreases the OXPHOS, suggesting that the proneural subtype is more prone to glycolysis than mesenchymal subtype. Thus, in a tumor progression setting from non-mesenchymal to more mesenchymal subtype glioblastoma cells, cells would be predicted to shift to OXPHOS. Interestingly, PROX1 has also been shown to regulate the gene expression of metabolic clock genes, and the perturbed clock is linked to glioma [255]. Further research on energy metabolism, and metabolic clock control, with the events of subtype transition should be considered.

Several pathways have been shown to regulate the cell cycle in neural precursors including Notch, Wnt, PDGF, Fibroblast growth factor (FGF) and Sonic hedgehog (SHH) signaling [277]. Notch1 binds with its ligand Jagged 1 and promotes proliferation of neural precursors [278]. Wnt signaling can upregulate cyclinD1 and shorten cell cycle in neural precursors

[279]. SHH binds with Ptc receptor and upregulates Gli and Bmi1 to promote neural precursors proliferation [280]. Interestingly, Notch regulator Numb has also been reported to suppress SHH signaling by targeting Gli [281]. FGF and their receptors, such as FGF2, FGF15, and FGF receptor 2 have also been shown to shorten cell cycle and increase proliferation [282]. In paper III, cell-to-cell interaction was shown to affect cell proliferation, where U-343 MGα C12:6 proliferated faster when growing on top of U-343 MG. However, NOTCH1 had an inhibitory effect since CRISPR-Cas9 knockout or shRNA suppression of NOTCH1 enhanced proliferation. This raises the question of the proliferation regulatory role of NOTCH1 in glioblastomas in a setting with intratumoral heterogeneity. Further studies also need be conducted to identify the proliferation-promoting factors that were masked by NOTCH1.

Further, the cell communication mediated by secreted factors was also found in the U-343 system, where a large number of secreted proteins were identified by secretome analysis, as examples, TGFBI and ADAMTS1 were tested and acted as suppressors on the proliferation of U-343 MGα 31L and secreted by U-343 MG. Further secreted factors were identified but not tested since the effect on proliferation by secreted factors in the U-343 model system appeared less potent than the cell-to-cell contact effect. It should also be noted that the cell-to-cell effects might be context dependent. Therefore it is suggested that interaction effects are also assessed in other models systems, as for example in neurosphere growth conditions and *in vivo* by mouse or zebrafish models. Our efforts can thus be seen to have defined models that now can be tested in these other systems. Also the SPECS method was found to be very powerful to identify secreted proteins without interference from the serum, and may be suitable to apply and develop for other models and systems. The correlation analysis between proteomics data and RNA-seq data provides a useable method for identification of relevant proteins.

### 8.3.3 The interaction with the tumor microenvironment

In paper I, we performed gene expression analysis of macrophage, pericyte and vascular markers in TCGA samples and noticed elevated expression of these in cases with a *SFRP2*<sup>high</sup>*SOX2*<sup>low</sup> expression pattern, and in which most of the cases belonged to mesenchymal subtype. Moreover, spatial gene expression analysis indicated a *SFRP2* signature in vascular tumor areas, whereas a *SOX2* signature presents more cellular areas. This suggests that mesenchymal tumor cells in vascular areas may attract immune cells. This is consistent with previous reports where M2 macrophages were found enriched in mesenchymal subtype glioblastomas [41]. By the addition of conditioned media from cells with *SFRP2* overexpression as compared to the native cells, CD206 expression was higher induced on human macrophages, suggesting a M2 polarization. However, further studies need to be conducted to identify the relevance and mechanism by which *SFRP2* induced this effect, direct effect on the macrophages, or via a secondary secreted factors from the tumor cells as for example CXCL12. CXCL12 was higher expressed in the tumor cells upon *SFRP2*

overexpression. The CXCL12-CXCR4 axis has previously been shown to be important for immune cell recruitment in glioblastoma [283].

The reason for why non-mesenchymal and mesenchymal subtype glioblastoma cells have different spatial distribution is not well studied. In this thesis, we noticed that the mesenchymal cells have stronger invasive capacity and mesenchymal genes expression based on *in vitro* Matrigel invasion assay and RNA-seq data. We also showed overexpression of SFRP2 and SOX2 respectively increased and decreased cell invasion (paper I). It could be vessel components/cells/microenvironment that shift the cancer cells to get mesenchymal features. However, we did not show evidence if this mesenchymal transition is plastic and reversible. Further experiments will be performed to see if mesenchymal cell state induced by SFRP2 could change back to original non-mesenchymal state after removal of SFRP2.

#### **8.3.4 The effect of intratumoral heterogeneity on drug resistance in glioblastoma**

In general, there is no single phenotype that can explain appearance of drug resistance. It is context dependent with regard to what drug you mean. In general, cytostatic drugs, which TMZ belongs to, are drugs that have an antiproliferative effect and preferentially kills dividing cells. The mechanism that allows cells to initially display or develop resistance to this type of drug may be very different as compared towards more novel targeted therapies, as for example EGFR inhibitors. In a perspective of intratumoral heterogeneity theoretically the main affecter is the heterogeneity in itself, with populations of cells with distinctly different phenotypes, which may range from differences in differentiation markers to mutations in different cancer driver genes. The heterogeneity may also present itself in more abstract phenotypes as for example drug sensitivity, with respect to still not yet known confounders. Here, the effect of heterogeneity on TMZ response was modeled in our U-343 system that pose as a suitable system to model appearance of drug resistance in glioblastoma. Still, given the extensive heterogeneity is also found between individual glioblastoma cases, further similar cell models needs to be generated and studied to get a representative repertoire of glioblastoma subgroups. In paper III, we analyzed cell ratios of each U-343 cell line when grown in mixture with the other U-343 cells with or without the presence of TMZ. Clearly, different cell lines have distinct inherent sensitivities to TMZ treatment. One cell line decreased to a small percentage of the whole co-cultures and would thus be difficult to detect in a corresponding initial tumor, however upon TMZ treatment it continues to proliferate and becomes the dominant population and thus could represent a cell population that cause tumor relapse after treatment. It is also striking how short time is needed to observe a clonal selection. The U-343 cells lines are relatively easy to culture with robust proliferation rate. Still, after only 10 days of culturing, the cells constitute only 1% of the cell populations. This model highlights a potential mechanism of treatment failure and appearance of a resistant drug population. To overcome this, in the case of drugs that resistance will be developed against, it is suggested to investigate combinatorial treatment approaches.

In summary, this thesis investigates causes and effects of intratumoral heterogeneity in glioblastoma during tumor evolution and progression. That is, non-mesenchymal cells in vascular areas are affected by SFRP2 that leads to a cascade of increased KLF4 levels, decreased AKT-phosphorylation, SOX2 and PROX1 levels, and decreased cell cycle proteins and proliferation, as well as transition to a mesenchymal gene expression signature. Within the tumor, the non-mesenchymal and mesenchymal cells may affect each other's growth, invasion, subtype states and drug sensitivity, resulting in treatment failure where the less sensitive population has taken over in the relapsed tumor. Therefore, we suggest that drugs that inhibit mesenchymal transition in combination with anti-proliferative drugs should be investigated as novel treatment strategies.





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## 10. REFERENCES

- 1 Ostrom QT, Gittleman H, Liao P, Vecchione-Koval T, Wolinsky Y, Kruchko C *et al.* CBTRUS Statistical Report: Primary brain and other central nervous system tumors diagnosed in the United States in 2010-2014. *Neuro Oncol* 2017; 19: v1-v88.
- 2 Yamanaka R, Hayano A, Kanayama T. Radiation-induced gliomas: a comprehensive review and meta-analysis. *Neurosurg Rev* 2018; 41: 719-731.
- 3 Zong H, Verhaak RG, Canoll P. The cellular origin for malignant glioma and prospects for clinical advancements. *Expert Rev Mol Diagn* 2012; 12: 383-394.
- 4 Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK *et al.* The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol* 2016; 131: 803-820.
- 5 Ohgaki H, Kleihues P. The definition of primary and secondary glioblastoma. *Clin Cancer Res* 2013; 19: 764-772.
- 6 Wang Y, Yang J, Zheng H, Tomasek GJ, Zhang P, McKeever PE *et al.* Expression of mutant p53 proteins implicates a lineage relationship between neural stem cells and malignant astrocytic glioma in a murine model. *Cancer Cell* 2009; 15: 514-526.
- 7 Jacques TS, Swales A, Brzozowski MJ, Henriquez NV, Linehan JM, Mirzadeh Z *et al.* Combinations of genetic mutations in the adult neural stem cell compartment determine brain tumour phenotypes. *EMBO J* 2010; 29: 222-235.
- 8 Uhrbom L, Dai C, Celestino JC, Rosenblum MK, Fuller GN, Holland EC. Ink4a-Arf loss cooperates with KRas activation in astrocytes and neural progenitors to generate glioblastomas of various morphologies depending on activated Akt. *Cancer Res* 2002; 62: 5551-5558.
- 9 Gladson CL, Prayson RA, Liu WM. The pathobiology of glioma tumors. *Annu Rev Pathol* 2010; 5: 33-50.
- 10 Lukas RV, Wainwright DA, Ladomersky E, Sachdev S, Sonabend AM, Stupp R. Newly Diagnosed Glioblastoma: A Review on Clinical Management. *Oncology (Williston Park)* 2019; 33: 91-100.
- 11 Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005; 352: 987-996.
- 12 Choi S, Yu Y, Grimmer MR, Wahl M, Chang SM, Costello JF. Temozolomide-associated hypermutation in gliomas. *Neuro Oncol* 2018; 20: 1300-1309.
- 13 Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R *et al.* Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. *N Engl J Med* 2014; 370: 709-722.
- 14 Wirsching HG, Tabatabai G, Roelcke U, Hottinger AF, Jorger F, Schmid A *et al.* Bevacizumab plus hypofractionated radiotherapy versus radiotherapy alone in elderly patients with glioblastoma: the randomized, open-label, phase II ARTE trial. *Ann Oncol* 2018; 29: 1423-1430.
- 15 Stupp R, Taillibert S, Kanner A, Read W, Steinberg D, Lhermitte B *et al.* Effect of Tumor-Treating Fields Plus Maintenance Temozolomide vs Maintenance Temozolomide Alone on Survival in Patients With Glioblastoma: A Randomized Clinical Trial. *JAMA* 2017; 318: 2306-2316.
- 16 Taphoorn MJB, Dirven L, Kanner AA, Lavy-Shahaf G, Weinberg U, Taillibert S *et al.* Influence of Treatment With Tumor-Treating Fields on Health-Related Quality of Life of Patients With Newly Diagnosed Glioblastoma: A Secondary Analysis of a Randomized Clinical Trial. *JAMA Oncol* 2018; 4: 495-504.
- 17 Inda MM, Fan X, Munoz J, Perot C, Fauvet D, Danglot G *et al.* Chromosomal abnormalities in human glioblastomas: gain in chromosome 7p correlating with loss in chromosome 10q. *Mol Carcinog* 2003; 36: 6-14.
- 18 Cancer Genome Atlas Research N. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008; 455: 1061-1068.
- 19 Lu C, Ward PS, Kapoor GS, Rohle D, Turcan S, Abdel-Wahab O *et al.* IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature* 2012; 483: 474-478.
- 20 Wang G, Sai K, Gong F, Yang Q, Chen F, Lin J. Mutation of isocitrate dehydrogenase 1 induces glioma cell proliferation via nuclear factor-kappaB activation in a hypoxia-inducible factor 1-alpha dependent manner. *Mol Med Rep* 2014; 9: 1799-1805.
- 21 Sasaki M, Knobbe CB, Itsumi M, Elia AJ, Harris IS, Chio, II *et al.* D-2-hydroxyglutarate produced by mutant IDH1 perturbs collagen maturation and basement membrane function. *Genes Dev* 2012; 26: 2038-2049.
- 22 Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W *et al.* IDH1 and IDH2 mutations in gliomas. *N Engl J Med* 2009; 360: 765-773.

- 23 Noushmehr H, Weisenberger DJ, Diefes K, Phillips HS, Pujara K, Berman BP *et al.* Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* 2010; 17: 510-522.
- 24 Nagpal J, Jamoona A, Gulati ND, Mohan A, Braun A, Murali R *et al.* Revisiting the role of p53 in primary and secondary glioblastomas. *Anticancer Res* 2006; 26: 4633-4639.
- 25 Kasthuber ER, Lowe SW. Putting p53 in Context. *Cell* 2017; 170: 1062-1078.
- 26 Lukashchuk N, Vousden KH. Ubiquitination and degradation of mutant p53. *Mol Cell Biol* 2007; 27: 8284-8295.
- 27 Ham SW, Jeon HY, Jin X, Kim EJ, Kim JK, Shin YJ *et al.* TP53 gain-of-function mutation promotes inflammation in glioblastoma. *Cell Death Differ* 2019; 26: 409-425.
- 28 Lee YR, Chen M, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor: new modes and prospects. *Nat Rev Mol Cell Biol* 2018; 19: 547-562.
- 29 Stambolic V, MacPherson D, Sas D, Lin Y, Snow B, Jang Y *et al.* Regulation of PTEN transcription by p53. *Mol Cell* 2001; 8: 317-325.
- 30 Mayo LD, Dixon JE, Durden DL, Tonks NK, Donner DB. PTEN protects p53 from Mdm2 and sensitizes cancer cells to chemotherapy. *J Biol Chem* 2002; 277: 5484-5489.
- 31 Li Y, Guessous F, Kwon S, Kumar M, Ibidapo O, Fuller L *et al.* PTEN has tumor-promoting properties in the setting of gain-of-function p53 mutations. *Cancer Res* 2008; 68: 1723-1731.
- 32 Hatanpaa KJ, Burma S, Zhao D, Habib AA. Epidermal growth factor receptor in glioma: signal transduction, neuropathology, imaging, and radioresistance. *Neoplasia* 2010; 12: 675-684.
- 33 Ramnarain DB, Park S, Lee DY, Hatanpaa KJ, Scoggin SO, Otu H *et al.* Differential gene expression analysis reveals generation of an autocrine loop by a mutant epidermal growth factor receptor in glioma cells. *Cancer Res* 2006; 66: 867-874.
- 34 Ozawa T, Brennan CW, Wang L, Squatrito M, Sasayama T, Nakada M *et al.* PDGFRA gene rearrangements are frequent genetic events in PDGFRA-amplified glioblastomas. *Genes Dev* 2010; 24: 2205-2218.
- 35 Killela PJ, Reitman ZJ, Jiao Y, Bettegowda C, Agrawal N, Diaz LA, Jr. *et al.* TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. *Proc Natl Acad Sci U S A* 2013; 110: 6021-6026.
- 36 Yuan Y, Qi C, Maling G, Xiang W, Yanhui L, Ruofei L *et al.* TERT mutation in glioma: Frequency, prognosis and risk. *J Clin Neurosci* 2016; 26: 57-62.
- 37 Heidenreich B, Rachakonda PS, Hemminki K, Kumar R. TERT promoter mutations in cancer development. *Curr Opin Genet Dev* 2014; 24: 30-37.
- 38 Wick W, Weller M, van den Bent M, Sanson M, Weiler M, von Deimling A *et al.* MGMT testing--the challenges for biomarker-based glioma treatment. *Nat Rev Neurol* 2014; 10: 372-385.
- 39 Hegi ME, Liu L, Herman JG, Stupp R, Wick W, Weller M *et al.* Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. *J Clin Oncol* 2008; 26: 4189-4199.
- 40 Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD *et al.* Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 2010; 17: 98-110.
- 41 Wang Q, Hu B, Hu X, Kim H, Squatrito M, Scarpace L *et al.* Tumor Evolution of Glioma-Intrinsic Gene Expression Subtypes Associates with Immunological Changes in the Microenvironment. *Cancer Cell* 2017; 32: 42-56 e46.
- 42 Cho DY, Przytycka TM. Dissecting cancer heterogeneity with a probabilistic genotype-phenotype model. *Nucleic Acids Res* 2013; 41: 8011-8020.
- 43 Nister M, Wedell B, Betsholtz C, Bywater M, Pettersson M, Westermarck B *et al.* Evidence for progression changes in the human malignant glioma line U-343 MGa: analysis of karyotype and expression of genes encoding the subunit chains of platelet-derived growth factor. *Cancer Res* 1987; 47: 4953-4960.
- 44 Visvader JE, Lindeman GJ. Cancer stem cells: current status and evolving complexities. *Cell Stem Cell* 2012; 10: 717-728.
- 45 Segerman A, Niklasson M, Haglund C, Bergstrom T, Jarvius M, Xie Y *et al.* Clonal Variation in Drug and Radiation Response among Glioma-Initiating Cells Is Linked to Proneural-Mesenchymal Transition. *Cell Rep* 2016; 17: 2994-3009.
- 46 Suva ML, Rheinbay E, Gillespie SM, Patel AP, Wakimoto H, Rabkin SD *et al.* Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. *Cell* 2014; 157: 580-594.

- 47 Joshi K, Banasavadi-Siddegowda Y, Mo X, Kim SH, Mao P, Kig C *et al.* MELK-dependent FOXM1 phosphorylation is essential for proliferation of glioma stem cells. *Stem Cells* 2013; 31: 1051-1063.
- 48 Gong AH, Wei P, Zhang S, Yao J, Yuan Y, Zhou AD *et al.* FoxM1 Drives a Feed-Forward STAT3-Activation Signaling Loop That Promotes the Self-Renewal and Tumorigenicity of Glioblastoma Stem-like Cells. *Cancer Res* 2015; 75: 2337-2348.
- 49 Yu OM, Benitez JA, Plouffe SW, Ryback D, Klein A, Smith J *et al.* YAP and MRTF-A, transcriptional co-activators of RhoA-mediated gene expression, are critical for glioblastoma tumorigenicity. *Oncogene* 2018; 37: 5492-5507.
- 50 Dong Z, Zhang G, Qu M, Gimple RC, Wu Q, Qiu Z *et al.* Targeting Glioblastoma Stem Cells through Disruption of the Circadian Clock. *Cancer Discov* 2019; 9: 1556-1573.
- 51 Lu R, Wang P, Parton T, Zhou Y, Chrysovergis K, Rockowitz S *et al.* Epigenetic Perturbations by Arg882-Mutated DNMT3A Potentiate Aberrant Stem Cell Gene-Expression Program and Acute Leukemia Development. *Cancer Cell* 2016; 30: 92-107.
- 52 Johnson KC, Houseman EA, King JE, von Herrmann KM, Fadul CE, Christensen BC. 5-Hydroxymethylcytosine localizes to enhancer elements and is associated with survival in glioblastoma patients. *Nat Commun* 2016; 7: 13177.
- 53 Kim E, Kim M, Woo DH, Shin Y, Shin J, Chang N *et al.* Phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. *Cancer Cell* 2013; 23: 839-852.
- 54 Cui Q, Shi H, Ye P, Li L, Qu Q, Sun G *et al.* m(6)A RNA Methylation Regulates the Self-Renewal and Tumorigenesis of Glioblastoma Stem Cells. *Cell Rep* 2017; 18: 2622-2634.
- 55 Marques-Torres MA, Gangoso E, Pollard SM. Modelling glioblastoma tumour-host cell interactions using adult brain organotypic slice co-culture. *Dis Model Mech* 2018; 11.
- 56 Charles N, Ozawa T, Squatrito M, Bleau AM, Brennan CW, Hambardzumyan D *et al.* Perivascular nitric oxide activates notch signaling and promotes stem-like character in PDGF-induced glioma cells. *Cell Stem Cell* 2010; 6: 141-152.
- 57 Ho IAW, Shim WSN. Contribution of the Microenvironmental Niche to Glioblastoma Heterogeneity. *Biomed Res Int* 2017; 2017: 9634172.
- 58 Zhu TS, Costello MA, Talsma CE, Flack CG, Crowley JG, Hamm LL *et al.* Endothelial cells create a stem cell niche in glioblastoma by providing NOTCH ligands that nurture self-renewal of cancer stem-like cells. *Cancer Res* 2011; 71: 6061-6072.
- 59 Yan GN, Yang L, Lv YF, Shi Y, Shen LL, Yao XH *et al.* Endothelial cells promote stem-like phenotype of glioma cells through activating the Hedgehog pathway. *J Pathol* 2014; 234: 11-22.
- 60 Cheng L, Huang Z, Zhou W, Wu Q, Donnola S, Liu JK *et al.* Glioblastoma stem cells generate vascular pericytes to support vessel function and tumor growth. *Cell* 2013; 153: 139-152.
- 61 Eyler CE, Wu Q, Yan K, MacSwords JM, Chandler-Militello D, Misuraca KL *et al.* Glioma stem cell proliferation and tumor growth are promoted by nitric oxide synthase-2. *Cell* 2011; 146: 53-66.
- 62 Lathia JD, Li M, Hall PE, Gallagher J, Hale JS, Wu Q *et al.* Laminin alpha 2 enables glioblastoma stem cell growth. *Ann Neurol* 2012; 72: 766-778.
- 63 Renner G, Janouskova H, Noulet F, Koenig V, Guerin E, Bar S *et al.* Integrin alpha5beta1 and p53 convergent pathways in the control of anti-apoptotic proteins PEA-15 and survivin in high-grade glioma. *Cell Death Differ* 2016; 23: 640-653.
- 64 Klingener M, Chavali M, Singh J, McMillan N, Coomes A, Dempsey PJ *et al.* N-cadherin promotes recruitment and migration of neural progenitor cells from the SVZ neural stem cell niche into demyelinated lesions. *J Neurosci* 2014; 34: 9590-9606.
- 65 Noh MG, Oh SJ, Ahn EJ, Kim YJ, Jung TY, Jung S *et al.* Prognostic significance of E-cadherin and N-cadherin expression in Gliomas. *BMC Cancer* 2017; 17: 583.
- 66 Eckerich C, Zapf S, Fillbrandt R, Loges S, Westphal M, Lamszus K. Hypoxia can induce c-Met expression in glioma cells and enhance SF/HGF-induced cell migration. *Int J Cancer* 2007; 121: 276-283.
- 67 Seidel S, Garvalov BK, Wirta V, von Stechow L, Schanzer A, Meletis K *et al.* A hypoxic niche regulates glioblastoma stem cells through hypoxia inducible factor 2 alpha. *Brain* 2010; 133: 983-995.
- 68 Gordan JD, Thompson CB, Simon MC. HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer Cell* 2007; 12: 108-113.
- 69 Heddlestone JM, Li Z, McLendon RE, Hjelmeland AB, Rich JN. The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype. *Cell Cycle* 2009; 8: 3274-3284.

- 70 Persano L, Rampazzo E, Basso G, Viola G. Glioblastoma cancer stem cells: role of the microenvironment and therapeutic targeting. *Biochem Pharmacol* 2013; 85: 612-622.
- 71 van Neerven SM, Tieken M, Vermeulen L, Bijlsma MF. Bidirectional interconversion of stem and non-stem cancer cell populations: A reassessment of theoretical models for tumor heterogeneity. *Mol Cell Oncol* 2016; 3: e1098791.
- 72 Friedmann-Morvinski D, Bushong EA, Ke E, Soda Y, Marumoto T, Singer O *et al.* Dedifferentiation of neurons and astrocytes by oncogenes can induce gliomas in mice. *Science* 2012; 338: 1080-1084.
- 73 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; 126: 663-676.
- 74 Piccirillo SG, Reynolds BA, Zanetti N, Lamorte G, Binda E, Broggi G *et al.* Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. *Nature* 2006; 444: 761-765.
- 75 Auffinger B, Tobias AL, Han Y, Lee G, Guo D, Dey M *et al.* Conversion of differentiated cancer cells into cancer stem-like cells in a glioblastoma model after primary chemotherapy. *Cell Death Differ* 2014; 21: 1119-1131.
- 76 Bonavia R, Inda MM, Cavenee WK, Furnari FB. Heterogeneity maintenance in glioblastoma: a social network. *Cancer Res* 2011; 71: 4055-4060.
- 77 Snuderl M, Fazlollahi L, Le LP, Nitta M, Zhelyazkova BH, Davidson CJ *et al.* Mosaic amplification of multiple receptor tyrosine kinase genes in glioblastoma. *Cancer Cell* 2011; 20: 810-817.
- 78 Szerlip NJ, Pedraza A, Chakravarty D, Azim M, McGuire J, Fang Y *et al.* Intratumoral heterogeneity of receptor tyrosine kinases EGFR and PDGFRA amplification in glioblastoma defines subpopulations with distinct growth factor response. *Proc Natl Acad Sci U S A* 2012; 109: 3041-3046.
- 79 Ozawa T, Riester M, Cheng YK, Huse JT, Squatrito M, Helmy K *et al.* Most human non-GCIMP glioblastoma subtypes evolve from a common proneural-like precursor glioma. *Cancer Cell* 2014; 26: 288-300.
- 80 Sottoriva A, Spiteri I, Piccirillo SG, Touloumis A, Collins VP, Marioni JC *et al.* Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc Natl Acad Sci U S A* 2013; 110: 4009-4014.
- 81 Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, Wakimoto H *et al.* Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* 2014; 344: 1396-1401.
- 82 Meyer M, Reimand J, Lan X, Head R, Zhu X, Kushida M *et al.* Single cell-derived clonal analysis of human glioblastoma links functional and genomic heterogeneity. *Proc Natl Acad Sci U S A* 2015; 112: 851-856.
- 83 Gupta PB, Fillmore CM, Jiang G, Shapira SD, Tao K, Kuperwasser C *et al.* Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell* 2011; 146: 633-644.
- 84 Piao Y, Liang J, Holmes L, Henry V, Sulman E, de Groot JF. Acquired resistance to anti-VEGF therapy in glioblastoma is associated with a mesenchymal transition. *Clin Cancer Res* 2013; 19: 4392-4403.
- 85 Fedele M, Cerchia L, Pegoraro S, Sgarra R, Manfioletti G. Proneural-Mesenchymal Transition: Phenotypic Plasticity to Acquire Multitherapy Resistance in Glioblastoma. *Int J Mol Sci* 2019; 20.
- 86 Carro MS, Lim WK, Alvarez MJ, Bollo RJ, Zhao X, Snyder EY *et al.* The transcriptional network for mesenchymal transformation of brain tumours. *Nature* 2010; 463: 318-325.
- 87 Bhat KP, Salazar KL, Balasubramanian V, Wani K, Heathcock L, Hollingsworth F *et al.* The transcriptional coactivator TAZ regulates mesenchymal differentiation in malignant glioma. *Genes Dev* 2011; 25: 2594-2609.
- 88 Halliday J, Helmy K, Pattwell SS, Pitter KL, LaPlant Q, Ozawa T *et al.* In vivo radiation response of proneural glioma characterized by protective p53 transcriptional program and proneural-mesenchymal shift. *Proc Natl Acad Sci U S A* 2014; 111: 5248-5253.
- 89 Lau J, Ilkhanizadeh S, Wang S, Miroshnikova YA, Salvatierra NA, Wong RA *et al.* STAT3 Blockade Inhibits Radiation-Induced Malignant Progression in Glioma. *Cancer Res* 2015; 75: 4302-4311.
- 90 Bhat KP, Balasubramanian V, Vaillant B, Ezhilarasan R, Hummelink K, Hollingsworth F *et al.* Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. *Cancer Cell* 2013; 24: 331-346.
- 91 Yin J, Oh YT, Kim JY, Kim SS, Choi E, Kim TH *et al.* Transglutaminase 2 Inhibition Reverses Mesenchymal Transdifferentiation of Glioma Stem Cells by Regulating C/EBPbeta Signaling. *Cancer Res* 2017; 77: 4973-4984.
- 92 Alafate W, Li X, Zuo J, Zhang H, Xiang J, Wu W *et al.* Elevation of CXCL1 indicates poor prognosis and radioresistance by inducing mesenchymal transition in glioblastoma. *CNS Neurosci Ther* 2020; 26: 475-485.
- 93 Lu F, Chen Y, Zhao C, Wang H, He D, Xu L *et al.* Olig2-Dependent Reciprocal Shift in PDGF and EGF Receptor Signaling Regulates Tumor Phenotype and Mitotic Growth in Malignant Glioma. *Cancer Cell* 2016; 29: 669-683.



- 94 Johnson BE, Mazar T, Hong C, Barnes M, Aihara K, McLean CY *et al.* Mutational analysis reveals the origin and therapy-driven evolution of recurrent glioma. *Science* 2014; 343: 189-193.
- 95 Kim J, Lee IH, Cho HJ, Park CK, Jung YS, Kim Y *et al.* Spatiotemporal Evolution of the Primary Glioblastoma Genome. *Cancer Cell* 2015; 28: 318-328.
- 96 Axelrod R, Axelrod DE, Pienta KJ. Evolution of cooperation among tumor cells. *Proc Natl Acad Sci U S A* 2006; 103: 13474-13479.
- 97 Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. *Biochim Biophys Acta* 2010; 1805: 105-117.
- 98 Davis JB, Krishna SS, Abi Jomaa R, Duong CT, Espina V, Liotta LA *et al.* A new model isolates glioblastoma clonal interactions and reveals unexpected modes for regulating motility, proliferation, and drug resistance. *Sci Rep* 2019; 9: 17380.
- 99 Inda MM, Bonavia R, Mukasa A, Narita Y, Sah DW, Vandenberg S *et al.* Tumor heterogeneity is an active process maintained by a mutant EGFR-induced cytokine circuit in glioblastoma. *Genes Dev* 2010; 24: 1731-1745.
- 100 Zanca C, Villa GR, Benitez JA, Thorne AH, Koga T, D'Antonio M *et al.* Glioblastoma cellular cross-talk converges on NF-kappaB to attenuate EGFR inhibitor sensitivity. *Genes Dev* 2017; 31: 1212-1227.
- 101 El-Sehemy A, Selvadurai H, Ortin-Martinez A, Pokrajac N, Mamatjan Y, Tachibana N *et al.* Norrin mediates tumor-promoting and -suppressive effects in glioblastoma via Notch and Wnt. *J Clin Invest* 2020; 130: 3069-3086.
- 102 Morris LG, Kaufman AM, Gong Y, Ramaswami D, Walsh LA, Turcan S *et al.* Recurrent somatic mutation of FAT1 in multiple human cancers leads to aberrant Wnt activation. *Nat Genet* 2013; 45: 253-261.
- 103 Kong DS, Song SY, Kim DH, Joo KM, Yoo JS, Koh JS *et al.* Prognostic significance of c-Met expression in glioblastomas. *Cancer* 2009; 115: 140-148.
- 104 Shojaei F, Lee JH, Simmons BH, Wong A, Esparza CO, Plumlee PA *et al.* HGF/c-Met acts as an alternative angiogenic pathway in sunitinib-resistant tumors. *Cancer Res* 2010; 70: 10090-10100.
- 105 Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO *et al.* MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007; 316: 1039-1043.
- 106 Kim KH, Seol HJ, Kim EH, Rhee J, Jin HJ, Lee Y *et al.* Wnt/beta-catenin signaling is a key downstream mediator of MET signaling in glioblastoma stem cells. *Neuro Oncol* 2013; 15: 161-171.
- 107 Hai L, Zhang C, Li T, Zhou X, Liu B, Li S *et al.* Notch1 is a prognostic factor that is distinctly activated in the classical and proneural subtype of glioblastoma and that promotes glioma cell survival via the NF-kappaB(p65) pathway. *Cell Death Dis* 2018; 9: 158.
- 108 Gumbiner BM, Kim NG. The Hippo-YAP signaling pathway and contact inhibition of growth. *J Cell Sci* 2014; 127: 709-717.
- 109 Shao DD, Xue W, Krall EB, Bhutkar A, Piccioni F, Wang X *et al.* KRAS and YAP1 converge to regulate EMT and tumor survival. *Cell* 2014; 158: 171-184.
- 110 Lu DY, Leung YM, Huang SM, Wong KL. Bradykinin-induced cell migration and COX-2 production mediated by the bradykinin B1 receptor in glioma cells. *J Cell Biochem* 2010; 110: 141-150.
- 111 Oliveira MN, Pillat MM, Motaln H, Ulrich H, Lah TT. Kinin-B1 Receptor Stimulation Promotes Invasion and is Involved in Cell-Cell Interaction of Co-Cultured Glioblastoma and Mesenchymal Stem Cells. *Sci Rep* 2018; 8: 1299.
- 112 Ghosh P, Singh UN. Intercellular communication in rapidly proliferating and differentiated C6 glioma cells in culture. *Cell Biol Int* 1997; 21: 551-557.
- 113 McDonough WS, Johansson A, Joffe H, Giese A, Berens ME. Gap junction intercellular communication in gliomas is inversely related to cell motility. *Int J Dev Neurosci* 1999; 17: 601-611.
- 114 Potthoff AL, Heiland DH, Evert BO, Almeida FR, Behringer SP, Dolf A *et al.* Inhibition of Gap Junctions Sensitizes Primary Glioblastoma Cells for Temozolomide. *Cancers (Basel)* 2019; 11.
- 115 Suzhi Z, Liang T, Yuexia P, Lucy L, Xiaoting H, Yuan Z *et al.* Gap Junctions Enhance the Antiproliferative Effect of MicroRNA-124-3p in Glioblastoma Cells. *J Cell Physiol* 2015; 230: 2476-2488.
- 116 Nakano I, Garnier D, Minata M, Rak J. Extracellular vesicles in the biology of brain tumour stem cells--Implications for inter-cellular communication, therapy and biomarker development. *Semin Cell Dev Biol* 2015; 40: 17-26.
- 117 Yekula A, Yekula A, Muralidharan K, Kang K, Carter BS, Balaj L. Extracellular Vesicles in Glioblastoma Tumor Microenvironment. *Front Immunol* 2019; 10: 3137.
- 118 Wang J, Liu J, Sun G, Meng H, Wang J, Guan Y *et al.* Glioblastoma extracellular vesicles induce the tumour-promoting transformation of neural stem cells. *Cancer Lett* 2019; 466: 1-12.

- 119 Choi D, Montermini L, Kim DK, Meehan B, Roth FP, Rak J. The Impact of Oncogenic EGFRvIII on the Proteome of Extracellular Vesicles Released from Glioblastoma Cells. *Mol Cell Proteomics* 2018; 17: 1948-1964.
- 120 de Mooij T, Peterson TE, Evans J, McCutcheon B, Parney IF. Short non-coding RNA sequencing of glioblastoma extracellular vesicles. *J Neurooncol* 2020; 146: 253-263.
- 121 Shi R, Wang PY, Li XY, Chen JX, Li Y, Zhang XZ *et al.* Exosomal levels of miRNA-21 from cerebrospinal fluids associated with poor prognosis and tumor recurrence of glioma patients. *Oncotarget* 2015; 6: 26971-26981.
- 122 Spinelli C, Montermini L, Meehan B, Brisson AR, Tan S, Choi D *et al.* Molecular subtypes and differentiation programmes of glioma stem cells as determinants of extracellular vesicle profiles and endothelial cell-stimulating activities. *J Extracell Vesicles* 2018; 7: 1490144.
- 123 Pavlyukov MS, Yu H, Bastola S, Minata M, Shender VO, Lee Y *et al.* Apoptotic Cell-Derived Extracellular Vesicles Promote Malignancy of Glioblastoma Via Intercellular Transfer of Splicing Factors. *Cancer Cell* 2018; 34: 119-135 e110.
- 124 Ramakrishnan V, Xu B, Akers J, Nguyen T, Ma J, Dhawan S *et al.* Radiation-induced extracellular vesicle (EV) release of miR-603 promotes IGF1-mediated stem cell state in glioblastomas. *EBioMedicine* 2020; 55: 102736.
- 125 Finn OJ. A Believer's Overview of Cancer Immunosurveillance and Immunotherapy. *J Immunol* 2018; 200: 385-391.
- 126 Hambardzumyan D, Bergers G. Glioblastoma: Defining Tumor Niches. *Trends Cancer* 2015; 1: 252-265.
- 127 Hambardzumyan D, Gutmann DH, Kettenmann H. The role of microglia and macrophages in glioma maintenance and progression. *Nat Neurosci* 2016; 19: 20-27.
- 128 Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 2010; 330: 841-845.
- 129 Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci* 2007; 10: 1538-1543.
- 130 Bennett ML, Bennett FC, Liddelow SA, Ajami B, Zamanian JL, Fernhoff NB *et al.* New tools for studying microglia in the mouse and human CNS. *Proc Natl Acad Sci U S A* 2016; 113: E1738-1746.
- 131 Bowman RL, Klemm F, Akkari L, Pyonteck SM, Sevenich L, Quail DF *et al.* Macrophage Ontogeny Underlies Differences in Tumor-Specific Education in Brain Malignancies. *Cell Rep* 2016; 17: 2445-2459.
- 132 Feng X, Szulzewsky F, Yerevanian A, Chen Z, Heinzmann D, Rasmussen RD *et al.* Loss of CX3CR1 increases accumulation of inflammatory monocytes and promotes gliomagenesis. *Oncotarget* 2015; 6: 15077-15094.
- 133 Ye XZ, Xu SL, Xin YH, Yu SC, Ping YF, Chen L *et al.* Tumor-associated microglia/macrophages enhance the invasion of glioma stem-like cells via TGF-beta1 signaling pathway. *J Immunol* 2012; 189: 444-453.
- 134 Solga AC, Pong WW, Kim KY, Cimino PJ, Toonen JA, Walker J *et al.* RNA Sequencing of Tumor-Associated Microglia Reveals Ccl5 as a Stromal Chemokine Critical for Neurofibromatosis-1 Glioma Growth. *Neoplasia* 2015; 17: 776-788.
- 135 Hussain SF, Yang D, Suki D, Aldape K, Grimm E, Heimberger AB. The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses. *Neuro Oncol* 2006; 8: 261-279.
- 136 Lu-Emerson C, Snuderl M, Kirkpatrick ND, Goveia J, Davidson C, Huang Y *et al.* Increase in tumor-associated macrophages after antiangiogenic therapy is associated with poor survival among patients with recurrent glioblastoma. *Neuro Oncol* 2013; 15: 1079-1087.
- 137 Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF *et al.* CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med* 2013; 19: 1264-1272.
- 138 Quail DF, Bowman RL, Akkari L, Quick ML, Schuhmacher AJ, Huse JT *et al.* The tumor microenvironment underlies acquired resistance to CSF-1R inhibition in gliomas. *Science* 2016; 352: aad3018.
- 139 Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, Yang DS, Sun W, Qiao W *et al.* Incidence and prognostic impact of FoxP3+ regulatory T cells in human gliomas. *Clin Cancer Res* 2008; 14: 5166-5172.
- 140 Bengsch B, Wherry EJ. The importance of cooperation: partnerless NFAT induces T cell exhaustion. *Immunity* 2015; 42: 203-205.
- 141 Chang CH, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD *et al.* Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell* 2015; 162: 1229-1241.
- 142 Kim JE, Patel MA, Mangraviti A, Kim ES, Theodoros D, Velarde E *et al.* Combination Therapy with Anti-PD-1, Anti-TIM-3, and Focal Radiation Results in Regression of Murine Gliomas. *Clin Cancer Res* 2017; 23: 124-136.

- 143 Heiland DH, Haaker G, Delev D, Mercas B, Masalha W, Heynckes S *et al.* Comprehensive analysis of PD-L1 expression in glioblastoma multiforme. *Oncotarget* 2017; 8: 42214-42225.
- 144 Nduom EK, Wei J, Yaghi NK, Huang N, Kong LY, Gabrusiewicz K *et al.* PD-L1 expression and prognostic impact in glioblastoma. *Neuro Oncol* 2016; 18: 195-205.
- 145 Reardon DA, Gokhale PC, Klein SR, Ligon KL, Rodig SJ, Ramkissoon SH *et al.* Glioblastoma Eradication Following Immune Checkpoint Blockade in an Orthotopic, Immunocompetent Model. *Cancer Immunol Res* 2016; 4: 124-135.
- 146 Zeng J, See AP, Phallen J, Jackson CM, Belcaid Z, Ruzevick J *et al.* Anti-PD-1 blockade and stereotactic radiation produce long-term survival in mice with intracranial gliomas. *Int J Radiat Oncol Biol Phys* 2013; 86: 343-349.
- 147 Chang AL, Miska J, Wainwright DA, Dey M, Rivetta CV, Yu D *et al.* CCL2 Produced by the Glioma Microenvironment Is Essential for the Recruitment of Regulatory T Cells and Myeloid-Derived Suppressor Cells. *Cancer Res* 2016; 76: 5671-5682.
- 148 Crane CA, Ahn BJ, Han SJ, Parsa AT. Soluble factors secreted by glioblastoma cell lines facilitate recruitment, survival, and expansion of regulatory T cells: implications for immunotherapy. *Neuro Oncol* 2012; 14: 584-595.
- 149 Zhai L, Lodomersky E, Lauing KL, Wu M, Genet M, Gritsina G *et al.* Infiltrating T Cells Increase IDO1 Expression in Glioblastoma and Contribute to Decreased Patient Survival. *Clin Cancer Res* 2017; 23: 6650-6660.
- 150 Hanihara M, Kawataki T, Oh-Okita K, Mitsuka K, Nakao A, Kinouchi H. Synergistic antitumor effect with indoleamine 2,3-dioxygenase inhibition and temozolomide in a murine glioma model. *J Neurosurg* 2016; 124: 1594-1601.
- 151 Akiyama Y, Nonomura C, Ashizawa T, Iizuka A, Kondou R, Miyata H *et al.* The anti-tumor activity of the STAT3 inhibitor STX-0119 occurs via promotion of tumor-infiltrating lymphocyte accumulation in temozolomide-resistant glioblastoma cell line. *Immunol Lett* 2017; 190: 20-25.
- 152 Zong J, Keskinov AA, Shurin GV, Shurin MR. Tumor-derived factors modulating dendritic cell function. *Cancer Immunol Immunother* 2016; 65: 821-833.
- 153 Wang J, Liu P, Xin S, Wang Z, Li J. Nrf2 suppresses the function of dendritic cells to facilitate the immune escape of glioma cells. *Exp Cell Res* 2017; 360: 66-73.
- 154 Kobayashi EH, Suzuki T, Funayama R, Nagashima T, Hayashi M, Sekine H *et al.* Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat Commun* 2016; 7: 11624.
- 155 Oshita C, Takikawa M, Kume A, Miyata H, Ashizawa T, Iizuka A *et al.* Dendritic cell-based vaccination in metastatic melanoma patients: phase II clinical trial. *Oncol Rep* 2012; 28: 1131-1138.
- 156 Prins RM, Soto H, Konkankit V, Odesa SK, Eskin A, Yong WH *et al.* Gene expression profile correlates with T-cell infiltration and relative survival in glioblastoma patients vaccinated with dendritic cell immunotherapy. *Clin Cancer Res* 2011; 17: 1603-1615.
- 157 Garg AD, Vandenberg L, Koks C, Verschuere T, Boon L, Van Gool SW *et al.* Dendritic cell vaccines based on immunogenic cell death elicit danger signals and T cell-driven rejection of high-grade glioma. *Sci Transl Med* 2016; 8: 328ra327.
- 158 Horng S, Therattil A, Moyon S, Gordon A, Kim K, Argaw AT *et al.* Astrocytic tight junctions control inflammatory CNS lesion pathogenesis. *J Clin Invest* 2017; 127: 3136-3151.
- 159 Boccazzi M, Ceruti S. Where do you come from and what are you going to become, reactive astrocyte? *Stem Cell Investig* 2016; 3: 15.
- 160 Brandao M, Simon T, Critchley G, Giamas G. Astrocytes, the rising stars of the glioblastoma microenvironment. *Glia* 2019; 67: 779-790.
- 161 Chen W, Wang D, Du X, He Y, Chen S, Shao Q *et al.* Glioma cells escaped from cytotoxicity of temozolomide and vincristine by communicating with human astrocytes. *Med Oncol* 2015; 32: 43.
- 162 Mega A, Hartmark Nilsen M, Leiss LW, Tobin NP, Miletic H, Sleire L *et al.* Astrocytes enhance glioblastoma growth. *Glia* 2020; 68: 316-327.
- 163 Kim JK, Jin X, Sohn YW, Jin X, Jeon HY, Kim EJ *et al.* Tumoral RANKL activates astrocytes that promote glioma cell invasion through cytokine signaling. *Cancer Lett* 2014; 353: 194-200.
- 164 Shnaper S, Desbaillets I, Brown DA, Murat A, Migliaiavacca E, Schluep M *et al.* Elevated levels of MIC-1/GDF15 in the cerebrospinal fluid of patients are associated with glioblastoma and worse outcome. *Int J Cancer* 2009; 125: 2624-2630.
- 165 Priego N, Zhu L, Monteiro C, Mulders M, Wasilewski D, Bindeman W *et al.* STAT3 labels a subpopulation of reactive astrocytes required for brain metastasis. *Nat Med* 2018; 24: 1024-1035.

- 166 Chen Q, Boire A, Jin X, Valiente M, Er EE, Lopez-Soto A *et al.* Carcinoma-astrocyte gap junctions promote brain metastasis by cGAMP transfer. *Nature* 2016; 533: 493-498.
- 167 Watkins S, Robel S, Kimbrough IF, Robert SM, Ellis-Davies G, Sontheimer H. Disruption of astrocyte-vascular coupling and the blood-brain barrier by invading glioma cells. *Nat Commun* 2014; 5: 4196.
- 168 Jain RK, di Tomaso E, Duda DG, Loeffler JS, Sorensen AG, Batchelor TT. Angiogenesis in brain tumours. *Nat Rev Neurosci* 2007; 8: 610-622.
- 169 Folkins C, Shaked Y, Man S, Tang T, Lee CR, Zhu Z *et al.* Glioma tumor stem-like cells promote tumor angiogenesis and vasculogenesis via vascular endothelial growth factor and stromal-derived factor 1. *Cancer Res* 2009; 69: 7243-7251.
- 170 Wang R, Chadalavada K, Wilshire J, Kowalik U, Hovinga KE, Geber A *et al.* Glioblastoma stem-like cells give rise to tumour endothelium. *Nature* 2010; 468: 829-833.
- 171 Deshors P, Toulas C, Arnauduc F, Malric L, Siegfried A, Nicaise Y *et al.* Ionizing radiation induces endothelial transdifferentiation of glioblastoma stem-like cells through the Tie2 signaling pathway. *Cell Death Dis* 2019; 10: 816.
- 172 Pietras A, Katz AM, Ekstrom EJ, Wee B, Halliday JJ, Pitter KL *et al.* Osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem cell phenotypes and promotes aggressive tumor growth. *Cell Stem Cell* 2014; 14: 357-369.
- 173 McCoy MG, Nyanyo D, Hung CK, Goerger JP, W RZ, Williams RM *et al.* Endothelial cells promote 3D invasion of GBM by IL-8-dependent induction of cancer stem cell properties. *Sci Rep* 2019; 9: 9069.
- 174 Schaefer T, Lengerke C. SOX2 protein biochemistry in stemness, reprogramming, and cancer: the PI3K/AKT/SOX2 axis and beyond. *Oncogene* 2020; 39: 278-292.
- 175 Pan H, Schultz RM. Sox2 modulates reprogramming of gene expression in two-cell mouse embryos. *Biol Reprod* 2011; 85: 409-416.
- 176 Takemoto T, Uchikawa M, Yoshida M, Bell DM, Lovell-Badge R, Papaioannou VE *et al.* Tbx6-dependent Sox2 regulation determines neural or mesodermal fate in axial stem cells. *Nature* 2011; 470: 394-398.
- 177 Cavallaro M, Mariani J, Lancini C, Latorre E, Caccia R, Gullo F *et al.* Impaired generation of mature neurons by neural stem cells from hypomorphic Sox2 mutants. *Development* 2008; 135: 541-557.
- 178 Que J, Luo X, Schwartz RJ, Hogan BL. Multiple roles for Sox2 in the developing and adult mouse trachea. *Development* 2009; 136: 1899-1907.
- 179 Que J, Okubo T, Goldenring JR, Nam KT, Kurotani R, Morrissey EE *et al.* Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development* 2007; 134: 2521-2531.
- 180 Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP *et al.* Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 2005; 122: 947-956.
- 181 Hoffmann SA, Hos D, Kuspert M, Lang RA, Lovell-Badge R, Wegner M *et al.* Stem cell factor Sox2 and its close relative Sox3 have differentiation functions in oligodendrocytes. *Development* 2014; 141: 39-50.
- 182 Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S *et al.* Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. *Cell Stem Cell* 2011; 9: 317-329.
- 183 Carrasco-Garcia E, Moreno-Cugnon L, Garcia I, Borrás C, Revuelta M, Izeta A *et al.* SOX2 expression diminishes with ageing in several tissues in mice and humans. *Mech Ageing Dev* 2019; 177: 30-36.
- 184 Kreso A, Dick JE. Evolution of the cancer stem cell model. *Cell Stem Cell* 2014; 14: 275-291.
- 185 Boumahdi S, Driessens G, Lapouge G, Rorive S, Nassar D, Le Mercier M *et al.* SOX2 controls tumour initiation and cancer stem-cell functions in squamous-cell carcinoma. *Nature* 2014; 511: 246-250.
- 186 Zhu F, Qian W, Zhang H, Liang Y, Wu M, Zhang Y *et al.* SOX2 Is a Marker for Stem-like Tumor Cells in Bladder Cancer. *Stem Cell Reports* 2017; 9: 429-437.
- 187 Takeda K, Mizushima T, Yokoyama Y, Hirose H, Wu X, Qian Y *et al.* Sox2 is associated with cancer stem-like properties in colorectal cancer. *Sci Rep* 2018; 8: 17639.
- 188 Justilien V, Walsh MP, Ali SA, Thompson EA, Murray NR, Fields AP. The PRKCI and SOX2 oncogenes are coamplified and cooperate to activate Hedgehog signaling in lung squamous cell carcinoma. *Cancer Cell* 2014; 25: 139-151.
- 189 Basu-Roy U, Bayin NS, Rattanakorn K, Han E, Placantonakis DG, Mansukhani A *et al.* Sox2 antagonizes the Hippo pathway to maintain stemness in cancer cells. *Nat Commun* 2015; 6: 6411.

- 190 Wang S, Tie J, Wang R, Hu F, Gao L, Wang W *et al.* SOX2, a predictor of survival in gastric cancer, inhibits cell proliferation and metastasis by regulating PTEN. *Cancer Lett* 2015; 358: 210-219.
- 191 Gangemi RM, Griffiro F, Marubbi D, Perera M, Capra MC, Malatesta P *et al.* SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. *Stem Cells* 2009; 27: 40-48.
- 192 Bulstrode H, Johnstone E, Marques-Torrejon MA, Ferguson KM, Bressan RB, Blin C *et al.* Elevated FOXG1 and SOX2 in glioblastoma enforces neural stem cell identity through transcriptional control of cell cycle and epigenetic regulators. *Genes Dev* 2017; 31: 757-773.
- 193 Lopez-Bertoni H, Kotchetkov IS, Mihelson N, Lal B, Rui Y, Ames H *et al.* A Sox2:miR-486-5p Axis Regulates Survival of GBM Cells by Inhibiting Tumor Suppressor Networks. *Cancer Res* 2020; 80: 1644-1655.
- 194 Wang J, Xu SL, Duan JJ, Yi L, Guo YF, Shi Y *et al.* Invasion of white matter tracts by glioma stem cells is regulated by a NOTCH1-SOX2 positive-feedback loop. *Nat Neurosci* 2019; 22: 91-105.
- 195 Ikushima H, Todo T, Ino Y, Takahashi M, Miyazawa K, Miyazono K. Autocrine TGF-beta signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors. *Cell Stem Cell* 2009; 5: 504-514.
- 196 Lamour V, Henry A, Kroonen J, Nokin MJ, von Marschall Z, Fisher LW *et al.* Targeting osteopontin suppresses glioblastoma stem-like cell character and tumorigenicity in vivo. *Int J Cancer* 2015; 137: 1047-1057.
- 197 Suwala AK, Koch K, Rios DH, Aretz P, Uhlmann C, Ogorek I *et al.* Inhibition of Wnt/beta-catenin signaling downregulates expression of aldehyde dehydrogenase isoform 3A1 (ALDH3A1) to reduce resistance against temozolomide in glioblastoma in vitro. *Oncotarget* 2018; 9: 22703-22716.
- 198 Liu T, Xu H, Huang M, Ma W, Saxena D, Lustig RA *et al.* Circulating Glioma Cells Exhibit Stem Cell-like Properties. *Cancer Res* 2018; 78: 6632-6642.
- 199 Liu L, Michowski W, Inuzuka H, Shimizu K, Nihira NT, Chick JM *et al.* G1 cyclins link proliferation, pluripotency and differentiation of embryonic stem cells. *Nat Cell Biol* 2017; 19: 177-188.
- 200 Fujikawa A, Sugawara H, Tanaka T, Matsumoto M, Kuboyama K, Suzuki R *et al.* Targeting PTPRZ inhibits stem cell-like properties and tumorigenicity in glioblastoma cells. *Sci Rep* 2017; 7: 5609.
- 201 Echizenya S, Ishii Y, Kitazawa S, Tanaka T, Matsuda S, Watanabe E *et al.* Discovery of a new pyrimidine synthesis inhibitor eradicating glioblastoma-initiating cells. *Neuro Oncol* 2020; 22: 229-239.
- 202 Garcia AL, Udeh A, Kalahasty K, Hackam AS. A growing field: The regulation of axonal regeneration by Wnt signaling. *Neural Regen Res* 2018; 13: 43-52.
- 203 Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM *et al.* Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. *Nature* 2002; 417: 664-667.
- 204 Schambony A, Wedlich D. Wnt-5A/Ror2 regulate expression of XPAPC through an alternative noncanonical signaling pathway. *Dev Cell* 2007; 12: 779-792.
- 205 Lin K, Wang S, Julius MA, Kitajewski J, Moos M, Jr., Luyten FP. The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. *Proc Natl Acad Sci USA* 1997; 94: 11196-11200.
- 206 Wang S, Krinks M, Lin K, Luyten FP, Moos M, Jr. Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell* 1997; 88: 757-766.
- 207 Mii Y, Taira M. Secreted Frizzled-related proteins enhance the diffusion of Wnt ligands and expand their signalling range. *Development* 2009; 136: 4083-4088.
- 208 von Marschall Z, Fisher LW. Secreted Frizzled-related protein-2 (sFRP2) augments canonical Wnt3a-induced signaling. *Biochem Biophys Res Commun* 2010; 400: 299-304.
- 209 Bhatt PM, Malgor R. Wnt5a: a player in the pathogenesis of atherosclerosis and other inflammatory disorders. *Atherosclerosis* 2014; 237: 155-162.
- 210 Bikkavilli RK, Avasarala S, Van Scoyk M, Arcaroli J, Brzezinski C, Zhang W *et al.* Wnt7a is a novel inducer of beta-catenin-independent tumor-suppressive cellular senescence in lung cancer. *Oncogene* 2015; 34: 5317-5328.
- 211 Yoshioka S, King ML, Ran S, Okuda H, MacLean JA, 2nd, McAsey ME *et al.* WNT7A regulates tumor growth and progression in ovarian cancer through the WNT/beta-catenin pathway. *Mol Cancer Res* 2012; 10: 469-482.
- 212 Zheng H, Ying H, Wiedemeyer R, Yan H, Quayle SN, Ivanova EV *et al.* PLAGL2 regulates Wnt signaling to impede differentiation in neural stem cells and gliomas. *Cancer Cell* 2010; 17: 497-509.
- 213 Rheinbay E, Suva ML, Gillespie SM, Wakimoto H, Patel AP, Shahid M *et al.* An aberrant transcription factor network essential for Wnt signaling and stem cell maintenance in glioblastoma. *Cell Rep* 2013; 3: 1567-1579.

- 214 Roth W, Wild-Bode C, Platten M, Grimmel C, Melkonyan HS, Dichgans J *et al.* Secreted Frizzled-related proteins inhibit motility and promote growth of human malignant glioma cells. *Oncogene* 2000; 19: 4210-4220.
- 215 Ramachandran I, Ganapathy V, Gillies E, Fonseca I, Sureban SM, Houchen CW *et al.* Wnt inhibitory factor 1 suppresses cancer stemness and induces cellular senescence. *Cell Death Dis* 2014; 5: e1246.
- 216 Yue X, Lan F, Yang W, Yang Y, Han L, Zhang A *et al.* Interruption of beta-catenin suppresses the EGFR pathway by blocking multiple oncogenic targets in human glioma cells. *Brain Res* 2010; 1366: 27-37.
- 217 Kamino M, Kishida M, Kibe T, Ikoma K, Iijima M, Hirano H *et al.* Wnt-5a signaling is correlated with infiltrative activity in human glioma by inducing cellular migration and MMP-2. *Cancer Sci* 2011; 102: 540-548.
- 218 Yuen TJ, Silbereis JC, Griveau A, Chang SM, Daneman R, Fancy SPJ *et al.* Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. *Cell* 2014; 158: 383-396.
- 219 Tsai HH, Niu J, Munji R, Davalos D, Chang J, Zhang H *et al.* Oligodendrocyte precursors migrate along vasculature in the developing nervous system. *Science* 2016; 351: 379-384.
- 220 Kim Y, Kim KH, Lee J, Lee YA, Kim M, Lee SJ *et al.* Wnt activation is implicated in glioblastoma radioresistance. *Lab Invest* 2012; 92: 466-473.
- 221 Lan F, Yue X, Han L, Yuan X, Shi Z, Huang K *et al.* Antitumor effect of aspirin in glioblastoma cells by modulation of beta-catenin/T-cell factor-mediated transcriptional activity. *J Neurosurg* 2011; 115: 780-788.
- 222 Sareddy GR, Kesanakurti D, Kirti PB, Babu PP. Nonsteroidal anti-inflammatory drugs diclofenac and celecoxib attenuates Wnt/beta-catenin/Tcf signaling pathway in human glioblastoma cells. *Neurochem Res* 2013; 38: 2313-2322.
- 223 Huang SM, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA *et al.* Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* 2009; 461: 614-620.
- 224 Rattner A, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA *et al.* A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. *Proc Natl Acad Sci U S A* 1997; 94: 2859-2863.
- 225 Jin L, Cao Y, Yu G, Wang J, Lin X, Ge L *et al.* SFRP2 enhances the osteogenic differentiation of apical papilla stem cells by antagonizing the canonical WNT pathway. *Cell Mol Biol Lett* 2017; 22: 14.
- 226 Zhou M, Jiao L, Liu Y. sFRP2 promotes airway inflammation and Th17/Treg imbalance in COPD via Wnt/beta-catenin pathway. *Respir Physiol Neurobiol* 2019; 270: 103282.
- 227 Zeng X, Zhang Y, Xu H, Zhang T, Xue Y, An R. Secreted Frizzled Related Protein 2 Modulates Epithelial-Mesenchymal Transition and Stemness via Wnt/beta-Catenin Signaling in Choriocarcinoma. *Cell Physiol Biochem* 2018; 50: 1815-1831.
- 228 Huang Z, Li L, Wang J. Hypermethylation of SFRP2 as a potential marker for stool-based detection of colorectal cancer and precancerous lesions. *Dig Dis Sci* 2007; 52: 2287-2291.
- 229 Veeck J, Noetzel E, Bektas N, Jost E, Hartmann A, Knuchel R *et al.* Promoter hypermethylation of the SFRP2 gene is a high-frequent alteration and tumor-specific epigenetic marker in human breast cancer. *Mol Cancer* 2008; 7: 83.
- 230 Cheng YY, Yu J, Wong YP, Man EP, To KF, Jin VX *et al.* Frequent epigenetic inactivation of secreted frizzled-related protein 2 (SFRP2) by promoter methylation in human gastric cancer. *Br J Cancer* 2007; 97: 895-901.
- 231 Chung MT, Lai HC, Sytwu HK, Yan MD, Shih YL, Chang CC *et al.* SFRP1 and SFRP2 suppress the transformation and invasion abilities of cervical cancer cells through Wnt signal pathway. *Gynecol Oncol* 2009; 112: 646-653.
- 232 Kaur A, Webster MR, Marchbank K, Behera R, Ndoye A, Kugel CH, 3rd *et al.* sFRP2 in the aged microenvironment drives melanoma metastasis and therapy resistance. *Nature* 2016; 532: 250-254.
- 233 Kim H, Yoo S, Zhou R, Xu A, Bernitz JM, Yuan Y *et al.* Oncogenic role of SFRP2 in p53-mutant osteosarcoma development via autocrine and paracrine mechanism. *Proc Natl Acad Sci U S A* 2018; 115: E11128-E11137.
- 234 Lin H, Angeli M, Chung KJ, Ejimadu C, Rosa AR, Lee T. sFRP2 activates Wnt/beta-catenin signaling in cardiac fibroblasts: differential roles in cell growth, energy metabolism, and extracellular matrix remodeling. *Am J Physiol Cell Physiol* 2016; 311: C710-C719.
- 235 Brinkmann EM, Mattes B, Kumar R, Hagemann AI, Gradl D, Scholpp S *et al.* Secreted Frizzled-related Protein 2 (sFRP2) Redirects Non-canonical Wnt Signaling from Fz7 to Ror2 during Vertebrate Gastrulation. *J Biol Chem* 2016; 291: 13730-13742.
- 236 Sun Y, Zhu D, Chen F, Qian M, Wei H, Chen W *et al.* SFRP2 augments WNT16B signaling to promote therapeutic resistance in the damaged tumor microenvironment. *Oncogene* 2016; 35: 4321-4334.
- 237 Yamamura S, Kawakami K, Hirata H, Ueno K, Saini S, Majid S *et al.* Oncogenic functions of secreted Frizzled-related protein 2 in human renal cancer. *Mol Cancer Ther* 2010; 9: 1680-1687.

- 238 Siamakpour-Reihani S, Caster J, Bandhu Nepal D, Courtwright A, Hilliard E, Usary J *et al.* The role of calcineurin/NFAT in SFRP2 induced angiogenesis--a rationale for breast cancer treatment with the calcineurin inhibitor tacrolimus. *PLoS One* 2011; 6: e20412.
- 239 Montagner M, Bhome R, Hooper S, Chakravarty P, Qin X, Sufi J *et al.* Crosstalk with lung epithelial cells regulates Sfrp2-mediated latency in breast cancer dissemination. *Nat Cell Biol* 2020; 22: 289-296.
- 240 Fontenot E, Rossi E, Mumper R, Snyder S, Siamakpour-Reihani S, Ma P *et al.* A novel monoclonal antibody to secreted frizzled-related protein 2 inhibits tumor growth. *Mol Cancer Ther* 2013; 12: 685-695.
- 241 Majchrzak-Celinska A, Slocinska M, Barciszewska AM, Nowak S, Baer-Dubowska W. Wnt pathway antagonists, SFRP1, SFRP2, SOX17, and PPP2R2B, are methylated in gliomas and SFRP1 methylation predicts shorter survival. *J Appl Genet* 2016; 57: 189-197.
- 242 Han M, Wang S, Fritah S, Wang X, Zhou W, Yang N *et al.* Interfering with long non-coding RNA MIR22HG processing inhibits glioblastoma progression through suppression of Wnt/beta-catenin signalling. *Brain* 2020; 143: 512-530.
- 243 Elsir T, Smits A, Lindstrom MS, Nister M. Transcription factor PROX1: its role in development and cancer. *Cancer Metastasis Rev* 2012; 31: 793-805.
- 244 Cha B, Geng X, Mahamud MR, Zhang JY, Chen L, Kim W *et al.* Complementary Wnt Sources Regulate Lymphatic Vascular Development via PROX1-Dependent Wnt/beta-Catenin Signaling. *Cell Rep* 2018; 25: 571-584 e575.
- 245 Gauvrit S, Villasenor A, Strilic B, Kitchen P, Collins MM, Marin-Juez R *et al.* HHX is a transcriptional regulator of the VEGFC/FLT4/PROX1 signaling axis during vascular development. *Nat Commun* 2018; 9: 2704.
- 246 Cho H, Kim J, Ahn JH, Hong YK, Makinen T, Lim DS *et al.* YAP and TAZ Negatively Regulate Prox1 During Developmental and Pathologic Lymphangiogenesis. *Circ Res* 2019; 124: 225-242.
- 247 Risebro CA, Searles RG, Melville AA, Ehler E, Jina N, Shah S *et al.* Prox1 maintains muscle structure and growth in the developing heart. *Development* 2009; 136: 495-505.
- 248 Kivela R, Salmela I, Nguyen YH, Petrova TV, Koistinen HA, Wiener Z *et al.* The transcription factor Prox1 is essential for satellite cell differentiation and muscle fibre-type regulation. *Nat Commun* 2016; 7: 13124.
- 249 Lavado A, Oliver G. Prox1 expression patterns in the developing and adult murine brain. *Dev Dyn* 2007; 236: 518-524.
- 250 Torii M, Matsuzaki F, Osumi N, Kaibuchi K, Nakamura S, Casarosa S *et al.* Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. *Development* 1999; 126: 443-456.
- 251 Jacob A, Wust HM, Thalhammer JM, Frob F, Kuspert M, Reiprich S *et al.* The transcription factor prospero homeobox protein 1 is a direct target of SoxC proteins during developmental vertebrate neurogenesis. *J Neurochem* 2018; 146: 251-268.
- 252 Kaltezioti V, Kouroupi G, Oikonomaki M, Mantouvalou E, Stergiopoulos A, Charonis A *et al.* Prox1 regulates the notch1-mediated inhibition of neurogenesis. *PLoS Biol* 2010; 8: e1000565.
- 253 Karalay O, Doberauer K, Vadodaria KC, Knobloch M, Berti L, Miquelajauregui A *et al.* Prospero-related homeobox 1 gene (Prox1) is regulated by canonical Wnt signaling and has a stage-specific role in adult hippocampal neurogenesis. *Proc Natl Acad Sci U S A* 2011; 108: 5807-5812.
- 254 Bunk EC, Ertaylan G, Ortega F, Pavlou MA, Gonzalez Cano L, Stergiopoulos A *et al.* Prox1 Is Required for Oligodendrocyte Cell Identity in Adult Neural Stem Cells of the Subventricular Zone. *Stem Cells* 2016; 34: 2115-2129.
- 255 Dufour CR, Levasseur MP, Pham NH, Eichner LJ, Wilson BJ, Charest-Marcotte A *et al.* Genomic convergence among ERRalpha, PROX1, and BMAL1 in the control of metabolic clock outputs. *PLoS Genet* 2011; 7: e1002143.
- 256 Takeda Y, Jetten AM. Prospero-related homeobox 1 (Prox1) functions as a novel modulator of retinoic acid-related orphan receptors alpha- and gamma-mediated transactivation. *Nucleic Acids Res* 2013; 41: 6992-7008.
- 257 Lecompte S, Pasquetti G, Hermant X, Grenier-Boley B, Gonzalez-Gross M, De Henauw S *et al.* Genetic and molecular insights into the role of PROX1 in glucose metabolism. *Diabetes* 2013; 62: 1738-1745.
- 258 Ragusa S, Cheng J, Ivanov KI, Zangger N, Ceteci F, Bernier-Latmani J *et al.* PROX1 promotes metabolic adaptation and fuels outgrowth of Wnt(high) metastatic colon cancer cells. *Cell Rep* 2014; 8: 1957-1973.
- 259 Wiener Z, Hogstrom J, Hyvonen V, Band AM, Kallio P, Holopainen T *et al.* Prox1 promotes expansion of the colorectal cancer stem cell population to fuel tumor growth and ischemia resistance. *Cell Rep* 2014; 8: 1943-1956.
- 260 Hogstrom J, Heino S, Kallio P, Lahde M, Leppanen VM, Balboa D *et al.* Transcription Factor PROX1 Suppresses Notch Pathway Activation via the Nucleosome Remodeling and Deacetylase Complex in Colorectal Cancer Stem-like Cells. *Cancer Res* 2018; 78: 5820-5832.

- 261 Versmold B, Felsberg J, Mikeska T, Ehrentraut D, Kohler J, Hampl JA *et al.* Epigenetic silencing of the candidate tumor suppressor gene PROX1 in sporadic breast cancer. *Int J Cancer* 2007; 121: 547-554.
- 262 Akagami M, Kawada K, Kubo H, Kawada M, Takahashi M, Kaganai J *et al.* Transcriptional factor Prox1 plays an essential role in the antiproliferative action of interferon-gamma in esophageal cancer cells. *Ann Surg Oncol* 2011; 18: 3868-3877.
- 263 Takahashi M, Yoshimoto T, Shimoda M, Kono T, Koizumi M, Yazumi S *et al.* Loss of function of the candidate tumor suppressor prox1 by RNA mutation in human cancer cells. *Neoplasia* 2006; 8: 1003-1010.
- 264 Foskolou IP, Stellas D, Rozani I, Lavigne MD, Politis PK. Prox1 suppresses the proliferation of neuroblastoma cells via a dual action in p27-Kip1 and Cdc25A. *Oncogene* 2013; 32: 947-960.
- 265 Shimoda M, Takahashi M, Yoshimoto T, Kono T, Ikai I, Kubo H. A homeobox protein, prox1, is involved in the differentiation, proliferation, and prognosis in hepatocellular carcinoma. *Clin Cancer Res* 2006; 12: 6005-6011.
- 266 Chang TM, Hung WC. The homeobox transcription factor Prox1 inhibits proliferation of hepatocellular carcinoma cells by inducing p53-dependent senescence-like phenotype. *Cancer Biol Ther* 2013; 14: 222-229.
- 267 Shi C, Xu X. MiR-670-5p induces cell proliferation in hepatocellular carcinoma by targeting PROX1. *Biomed Pharmacother* 2016; 77: 20-26.
- 268 Liu Y, Zhang JB, Qin Y, Wang W, Wei L, Teng Y *et al.* PROX1 promotes hepatocellular carcinoma metastasis by way of up-regulating hypoxia-inducible factor 1alpha expression and protein stability. *Hepatology* 2013; 58: 692-705.
- 269 Liu Y, Ye X, Zhang JB, Ouyang H, Shen Z, Wu Y *et al.* PROX1 promotes hepatocellular carcinoma proliferation and sorafenib resistance by enhancing beta-catenin expression and nuclear translocation. *Oncogene* 2015; 34: 5524-5535.
- 270 Elsir T, Eriksson A, Orrego A, Lindstrom MS, Nister M. Expression of PROX1 Is a common feature of high-grade malignant astrocytic gliomas. *J Neuropathol Exp Neurol* 2010; 69: 129-138.
- 271 Elsir T, Qu M, Berntsson SG, Orrego A, Olofsson T, Lindstrom MS *et al.* PROX1 is a predictor of survival for gliomas WHO grade II. *Br J Cancer* 2011; 104: 1747-1754.
- 272 Roodakker KR, Elsir T, Edqvist PD, Hagerstrand D, Carlson J, Lysiak M *et al.* PROX1 is a novel pathway-specific prognostic biomarker for high-grade astrocytomas; results from independent glioblastoma cohorts stratified by age and IDH mutation status. *Oncotarget* 2016; 7: 72431-72442.
- 273 Xu X, Wan X, Wei X. PROX1 promotes human glioblastoma cell proliferation and invasion via activation of the nuclear factor-kappaB signaling pathway. *Mol Med Rep* 2017; 15: 963-968.
- 274 Wang Z, Kang L, Zhang H, Huang Y, Fang L, Li M *et al.* AKT drives SOX2 overexpression and cancer cell stemness in esophageal cancer by protecting SOX2 from UBR5-mediated degradation. *Oncogene* 2019; 38: 5250-5264.
- 275 Fasano CA, Dimos JT, Ivanova NB, Lowry N, Lemischka IR, Temple S. shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. *Cell Stem Cell* 2007; 1: 87-99.
- 276 Yoshida Y, Takahashi K, Okita K, Ichisaka T, Yamanaka S. Hypoxia enhances the generation of induced pluripotent stem cells. *Cell Stem Cell* 2009; 5: 237-241.
- 277 Salomoni P, Calegari F. Cell cycle control of mammalian neural stem cells: putting a speed limit on G1. *Trends Cell Biol* 2010; 20: 233-243.
- 278 Nyfeler Y, Kirch RD, Mantei N, Leone DP, Radtke F, Suter U *et al.* Jagged1 signals in the postnatal subventricular zone are required for neural stem cell self-renewal. *EMBO J* 2005; 24: 3504-3515.
- 279 Michaelidis TM, Lie DC. Wnt signaling and neural stem cells: caught in the Wnt web. *Cell Tissue Res* 2008; 331: 193-210.
- 280 Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S *et al.* Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. *Nat Neurosci* 2008; 11: 277-284.
- 281 Di Marcotullio L, Ferretti E, Greco A, De Smaele E, Po A, Sico MA *et al.* Numb is a suppressor of Hedgehog signalling and targets Gli1 for Itch-dependent ubiquitination. *Nat Cell Biol* 2006; 8: 1415-1423.
- 282 Iwata T, Hevner RF. Fibroblast growth factor signaling in development of the cerebral cortex. *Dev Growth Differ* 2009; 51: 299-323.
- 283 Wurth R, Bajetto A, Harrison JK, Barbieri F, Florio T. CXCL12 modulation of CXCR4 and CXCR7 activity in human glioblastoma stem-like cells and regulation of the tumor microenvironment. *Front Cell Neurosci* 2014; 8: 144.